VOLUME 8 November 1959 NUMBER 6

THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

Combining The American Journal of Tropical Medicine and The Journal of the National Malaria Society

OFFICIAL ORGAN OF
THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

PUBLISHED BIMONTHLY BY
THE WILLIAMS & WILKINS COMPANY
BALTIMORE 2, MARYLAND, U.S.A.

© Copyright 1959, By The American Society of Tropical Medicine and Hygiene
Made in U.S.A.

THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

Editor-in-Chief, pro tem
MARTIN D. YOUNG
P. O. Box 717
Columbia, South Carolina

Book Review and Issue Editor, pro tem
MARTIN FROBISHER
374 Ruckman Road
Closter, New Jersey

Editor, Tropical Medicine and Hygiene News
LOUIS J. OLIVIER, National Institutes of Health
Bethesda 14, Maryland

Editorial Board

MARTIN D. YOUNG, *Chairman*
PAUL C. BEAVER
WILLIAM McD. HAMMON
JOHN M. HENDERSON
HARRY MOST
LLOYD E. ROZEBOOM
ALBERT B. SABIN
LEON H. SCHMIDT

INFORMATION FOR SUBSCRIBERS

THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE is issued bimonthly, appearing in January, March, May, July, September, and November. One volume is issued per year.

Correspondence concerning business matters, subscriptions and orders for reprints, should be addressed to The Williams & Wilkins Company, 428 East Preston Street, Baltimore 2, Maryland, U. S. A.

Manuscripts and Books for Review should be sent to Dr. Martin Frobisher, 374 Ruckman Road, Closter, New Jersey.

New subscriptions and renewals are entered to begin with the first issue of the current volume. Should any issue of the current volume be out of print at the time the subscription order is received, the pro rata value of such numbers will be refunded to the subscriber.

Subscriptions should be renewed promptly—To avoid a break in your series, subscriptions should be renewed promptly. The publishers cannot guarantee to supply back issues on belated renewals.

Change of address: Publisher must be notified 60 days in advance. Journals undeliverable because of incorrect address will be destroyed. Duplicates can be obtained (if available) from the publisher at the regular price of single issues.

OFFICERS OF THE

American Society of Tropical Medicine and Hygiene

President	*Councilors:*	*Term Expires*
LEWIS W. HACKETT	LESLIE A. STAUBER	1959
President-Elect	WILLIAM C. REEVES	1959
WILLARD H. WRIGHT	JOHN M. HENDERSON	1960
Vice-President	J. ALLEN SCOTT	1960
OLIVER R. McCOY	PAUL F. RUSSELL	1961
Secretary-Treasurer	JOSEPH E. SMADEL	1961
ROLLA B. HILL	MARSHALL HERTIG	1962
3575 St. Gaudens Road	JOHN E. LARSH, JR.	1962
Miami 33, Florida		

Individuals interested in any phase of tropical medicine and hygiene, including all arthropod-borne diseases, medical care and human welfare are eligible for membership. Dues are $10.00 a year and include subscription to the American Journal of Tropical Medicine and Hygiene. An application blank is bound into this issue of the Journal for the use of interested individuals. Before being submitted to the Secretary, each application must be signed by two active members of the Society and a check for $10.00 (U.S. banks preferred) must accompany the application.

Published bimonthly by The Williams and Wilkins Co., 428 East Preston Street, Baltimore 2, Md., U.S.A.
Subscription price $10.00, single issue $3.00
Second-class mail privileges authorized at Baltimore, Md.

NEW ONE-DOSE TREATMENT FOR PINWORMS

Povan*

SUSPENSION

(PYRVINIUM PAMOATE SUSPENSION, PARKE-DAVIS)

A new agent, POVAN SUSPENSION is singularly effective against pinworms...greatly improves and simplifies therapy.

- single-dose effectiveness in pinworm infections[1]
- pleasant-tasting and well tolerated
- easy to administer and economical
- practical against the spread of oxyuriasis...a single dose to each member of a household or institution where pinworms are present[1]

Administration and Dosage

POVAN SUSPENSION is administered orally in a single dose. In small children, the dose is equivalent to 5 mg. pyrvinium base per Kg. of body weight.

For convenience, a 5-cc. teaspoonful per 22 pounds (10 Kg.) of body weight may be recommended. For example, a 54-pound child would receive somewhat less than 3 teaspoonfuls of the Suspension.

Adults also may be given POVAN SUSPENSION according to the same dosage schedule.

Note: Parents and patients should be informed that POVAN SUSPENSION will color the stools a bright red and that, if spilled, will stain.

Supplied: POVAN SUSPENSION is available as a pleasant-tasting, strawberry-flavored suspension containing the equivalent of 10 mg. pyrvinium base per cc., in 2-oz. bottles.

(1) Beck, J. W.; Saavedra, D.; Antell, G. J., & Tejeiro, B.: Am. J. Trop. Med. 8:349, 1959.

*TRADE-MARK

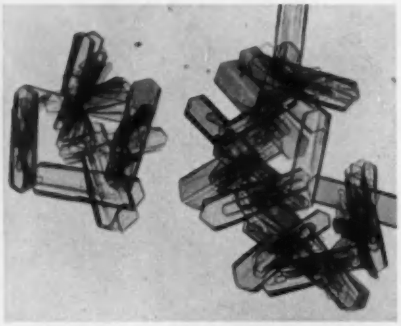
PARKE, DAVIS & COMPANY
DETROIT 32, MICHIGAN

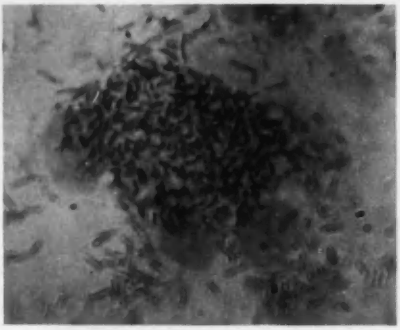
08459

AN IMPORTANT CONTRIBUTION TO THE TREATMENT OF LEPROSY...

CIBA-1906

CIBA-1906, A THIOUREA DERIVATIVE, HAS SHOWN THE FOLLOWING ADVANTAGES DURING 4 YEARS OF CLINICAL TRIAL IN OVER 20 COUNTRIES:





- reliable antibacterial action in both lepromatous and tuberculoid leprosy, even in patients unresponsive or resistant to other antileprosy drugs.
- minimum of toxic side effects; well tolerated even by children and patients hypersensitive to sulphones.
- produces prompt reduction in bacterial count, with correspondingly rapid clinical improvement.
- "lepra reactions" occur rarely and in mild form.
- less scar formation and nerve destruction.
- does not cause depression; may produce a sense of well-being.
- no known contraindications.
- can be combined with other antileprosy agents.

Available only for investigational use; for further information write to:
CIBA LIMITED, BASLE, SWITZERLAND

2/2748 HB

THE AMERICAN JOURNAL OF
TROPICAL MEDICINE AND HYGIENE

Vol. 8 *November 1959* *No. 6*

CONTENTS

Persistence of bacteria in the developmental stages of the housefly. III. Quantitative distribution in prepupae and pupae. BERNARD GREENBERG 613

Persistence of bacteria in the developmental stages of the housefly. IV. Infectivity of the newly emerged adult. BERNARD GREENBERG 618

Chloroquine compared with chloroquine and emetine combined in amebic liver abscess. A. J. WILMOT, S. J. POWELL AND E. B. ADAMS 623

Investigation of parasitic infections in the central area of Philadelphia. DANIEL WEINER, M. M. BROOKE, AND A. WITKOW 625

Relation of water velocity to *Schistosoma mansoni* infection in mice. WILLIAM B. ROWAN AND ANDREW L. GRAM 630

Malaria surveillance in the United States, 1958. JACOB A. BRODY AND FREDERICK L. DUNN 635

Intradermal tests with *Dirofilaria immitis* extract in eosinophilic lung (tropical eosinophilia). T. J. DANARAJ AND JOHN F. SCHACHER 640

Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. W. F. SCHERER AND E. L. BUESCHER 644

Ecologic studies of Japanese encephalitis virus in Japan. II. Mosquito infection. E. L. BUESCHER, W. F. SCHERER, M. Z. ROSENBERG, I. GRESSER, J. L. HARDY AND H. R. BULLOCK 651

Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors. Zootropism and vertical flight of *Culex tritaeniorhynchus* with observations on variations in collections from animal-baited traps in different habitats. W. F. SCHERER, E. L. BUESCHER, M. B. FLEMINGS, A. NOGUCHI AND J. SCANLON 665

Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. E. L. BUESCHER, W. F. SCHERER, H. E. MCCLURE, J. T. MOYER, M. Z. ROSENBERG, M. YOSHII AND Y. OKADA 678

Ecologic studies of Japanese encephalitis virus in Japan. V. Avian factors. W. F. SCHERER, E. L. BUESCHER AND H. E. MCCLURE 689

Ecologic studies of Japanese encephalitis virus in Japan. VI. Swine infection. W. F. SCHERER, J. T. MOYER, T. IZUMI, I. GRESSER AND J. MCCOWN 698

Ecologic studies of Japanese encephalitis virus in Japan. VII. Human infection. W. F. SCHERER, M. KITAOKA, T. OKUNO AND T. OGATA 707

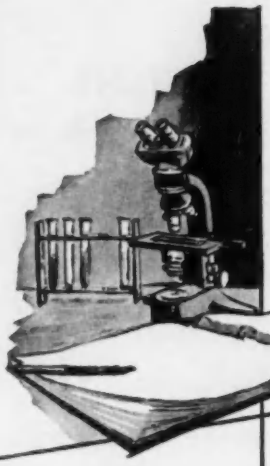
Ecologic studies of Japanese encephalitis virus in Japan. VIII. Survey for infection of wild rodents. W. F. SCHERER, E. L. BUESCHER, C. M. SOUTHAM, M. B. FLEMINGS AND A. NOGUCHI 716

Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemiologic correlations and conclusions. E. L. BUESCHER AND W. F. SCHERER 719

Recovery of herpes simplex, spontaneous murine and chick viruses in tests on specimens from infectious hepatitis patients. J. A. MORRIS AND K. NAKAMURA 723

Book reviews: *Perspectives in Virology*, by Pollard; *The Mammals of North America*, by Hall and Kelson; *Annals of the Institute of Tropical Medicine*, by various authors; *Antibiotics Annual 1958–1959*, by Welch and Marti-Ibanez 729

Correction 732



notes from a MICROSCOPIST'S NOTEBOOK

FAT STAINING

Techniques and solutions for the demonstration of fat in tissue have advanced markedly since Daddi's first oil soluble dye for this purpose in 1896.

Using alcohol as the solvent for the dye, early workers often dissolved out much of the fat present. Subsequently, solutions based on equal parts of 70% alcohol and acetone were used, and more recently solutions of 50% to 60% isopropanol and ethylene glycol or propylene glycol have found favor.

Advances in solutions have been paralleled by better techniques and the availability of a wider selection of fat stains which provide greater depth of color and contrast.

National has steadily expanded its line of Biological Stains and Indicators to include the principal stains in current use for demonstrating fat in tissue.

For your convenience, we list their Catalog Numbers:

#673—Sudan II	** #629—Sudan Black B*
** #635—Sudan III*	*#689—Oil Red O*
** #542—Sudan IV*	*#599—Oil Blue N*
	** #686—Nile Blue A*

*Certified by the Biological Stain Commission

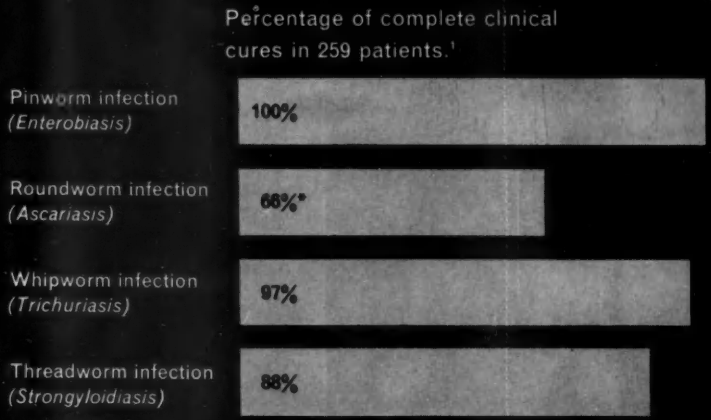
NATIONAL
BIOLOGICAL STAINS and INDICATORS

Allied Chemical

NATIONAL ANILINE DIVISION
40 RECTOR STREET, NEW YORK 6, N. Y.

eliminates four major worm infections

Lilly
QUALITY / RESEARCH / INTEGRITY



*Although one-third of the patients with roundworm infections were not completely cured, Delvex produced a 97 percent reduction in fecal egg counts.

New DELVEX™, the first wide-spectrum anthelmintic

Clinical studies show:

- Delvex is effective orally, usually within five days, against four of the five most common worm infections:
  Pinworm Whipworm
  Roundworm Strongyloidiasis
- It is partially effective, and sometimes curative, in hookworm infection.
- It is fully effective in both single and multiple infections and in both heavy and light infections.
- In optimal dosage, it eliminates pinworm infection in 100 percent of patients.
- It is the first effective and practicable agent for the oral treatment of strongyloidiasis and whipworm infection.
- No adjunctive measures are needed with Delvex therapy.

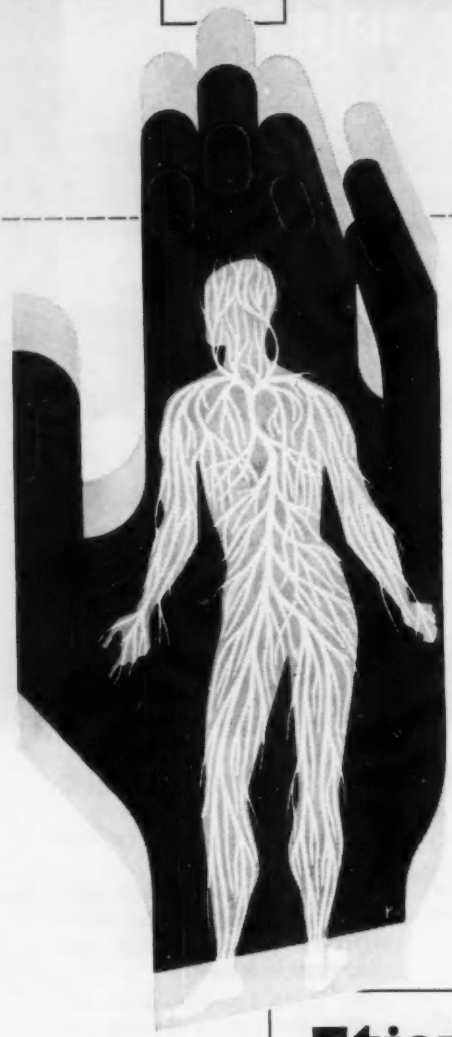
Further information and clinical reports may be obtained from your Lilly representative or by writing to our medical division.

1. Swartzwelder, J. C., et al.: J.A.M.A., 165:2063, 1957.

Delvex™ (dithiazanine iodide, Lilly)

ELI LILLY AND COMPANY • INDIANAPOLIS 6, INDIANA, U.S.A.
960618

The new ICI treatment for Leprosy



Advantages of 'Etisul'

1 Chemotherapeutic action —
Rapid and pronounced.

2 Safety —
Side effects minimal, regardless of age or sex of the patient.

3 Psychologically sound
in its method of administration.

4 Mass treatment —
DDS and 'Etisul' may be combined with advantage in mass treatment.

'Etisul' (percutaneous) is presented as a cream for inunction in unit packs containing 5 grammes of active agent.

Literature and further information available on request.

References: Lep. Rev. (1959), **1**, 61–72
Ibid. (1959), **3**, 141

Etisul Trade Mark (PERCUTANEOUS)

Diethyl-Dithiolisophthalate (I.C.I. Compound 15688)

IMPERIAL CHEMICAL INDUSTRIES LIMITED
Ph.942:0 PHARMACEUTICALS DIVISION WILMSLOW CHESHIRE ENGLAND

SMITH SPRAYERS

"For 70 Years Choice for Quality the World Over"

FOR MOSQUITO & MALARIA CONTROL

For Residual House Spraying or Larviciding Operations. Herculoy Silicon Bronze Sprayers are available in 4 gallon and smaller sizes and Armco Zinc-grip steel sprayers are also available in smaller sizes.

E-Z Knapsack Sprayer

HEAVY SEAMLESS BRASS PUMP QUICKLY REMOVED.
LARGE 4" OPENING FOR QUICK FILLING REMOVABLE BRASS STRAINER.
TRIGGER SHUT-OFF LOCKS OPEN.
BRASS ADJUSTABLE NOZZLE FOR FOG MIST, COARSE OR LONG DISTANCE SPRAY.
TUBULAR STEEL.
PISTON — HEAVY OIL-PROOF RUBBER FOR LONG SERVICE. EASILY REPLACED.
VENTILATED AND FORM FITTING BACK.
1-PIECE BRASS PUMP CUP.
TANK MADE OF ARMCO ZINC GRIP STEEL OR MANGANESE BRASS. ARMCO IS FAR SUPERIOR TO ANY GALVANIZED METAL.
E-Z
DASH AGITATOR
AIR-COOLED TANK
5-GALLON TANK

One in case.
Shipping weight—20 lbs.

Maintains continuous high pressure, with only easy slow pumping. Pump entirely of brass to stand long hard usage. Tank solid copper or Armco zinc-grip steel. Equipped with best grade, high-pressure hose, 2-foot brass extension pipe with adjustable brass or fixed type nozzle. New shut-off control may be locked open for continuous spray. Capacity 4 to 5 gallons. Has form-fitting ventilated tank, keeping the back dry and comfortable. Carrying handle; and dasher type agitator which keeps liquid thoroughly mixed. Brass liquid strainer.

●

MANY OTHER STYLES— SEND FOR CATALOG

on our complete line of hand, continuous, compressed air, knapsack, bucket, wheelbarrow and barrel sprayers. Also hand and crank powder insecticide dusters. We are the originators of sprayers and our products are of outstanding quality in engineering, modern design and performance.

D. B. SMITH & COMPANY, 432 MAIN ST., UTICA 2, NEW YORK, U. S. A.
ESTABLISHED 1888

PEAK PERFORMANCE AGAINST INSECT PESTS

TIFA

TODD INSECTICIDAL FOG APPLICATOR

OVER 14 YEARS Successful World-Wide Operation in Public Health Programs

FACTORY MAINTENANCE WINTERTIME REPAIRS · OVERHAULS!

Factory mechanics check, service or recondition your TIFA machines. Get ready for next season . . . *let experts* protect your investment! For details, contact either Brooklyn or Houston plant.

Peak performance against all insects pests... on the dump, over pools, in suburbs, play areas, everywhere insects swarm. Best by test for over 14 years, TIFA sets the records for lowest operating and maintenance costs in over 4067 communities throughout the world. True clean fog with maximum efficiency, using newest or older type insecticides (solution, emulsion, suspension) . . . one man operation.

Carrying Underwriters' Listing Seal No. MH 4162 *Write for illustrated folder and official comments*

TODD SHIPYARDS CORPORATION

PRODUCTS DIVISION

SALES AND SERVICE DEPARTMENTS
Columbia & Halleck Streets, Brooklyn 31, N. Y.

PLANT, SALES AND SERVICE
Houston, Texas

Sales & Service Agencies throughout the Western Hemisphere

THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

APPLICATION FOR MEMBERSHIP

To the Membership Committee:

The following individual interested in tropical medicine and hygiene is proposed for active membership

(TYPE OR PRINT)

Name..... (*Last*) (*First*) (*Middle*)

Mailing Address: Street..... City.....

State..... Country.....

Date of Birth..... Place.....

Present Position.....

Special Field of Interest.....

Education: Institution (Graduate and Postgraduate) Degree Date

.....

.....

.....

Attach to the application a list of professional positions held, membership in scientific societies, and a list of major publications.

* Proposed by:

(1) ....

(2) ..... Signature of Applicant

* Two active members must sign this application. Date.....

Return this application to Dr. Rolla B. Hill, Secretary-Treasurer, 3575 St. Gaudens Road, Miami 33, Florida

(*Check for $10.00 (U. S. banks requested) must accompany this application.*)

a rapid return to health with

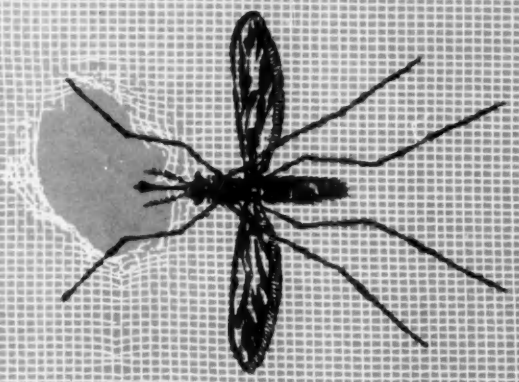
CAMOQUIN®

SINGLE-DOSE ANTIMALARIAL

A single three-tablet dose of CAMOQUIN hydrochloride (amodiaquin hydrochloride, Parke-Davis) reduces fever, clears the blood stream, and relieves other symptoms —often within 24 hours. Ideal for mass treatment because of its economy and fast action. CAMOQUIN is also valuable as a suppressive agent.

Supplied: In packages of 3 tablets of 0.2 Gm. each, and in bottles of 1000 tablets.

PARKE, DAVIS & COMPANY
DETROIT 32, MICHIGAN



Hastening the decline of malaria

'DARAPRIM'® brand
Pyrimethamine

"... the most efficient suppressive agent known, ..."

"... of extraordinary effectiveness against erythrocytic parasites of all the malarias."

"... probably has some action against the primary exo-erythrocytic forms of all the malarias, ..."

"... is active against the late tissue forms of *Plasmodium vivax* ..."

"... exhibits a phenomenal effect on gametocytes. ... the parasites are not able to develop beyond the early oöcyst stage, thereby blocking the chain of transmission."

Coatney, G. R. : Industry and Tropical Health, Volume II, 1955.

For the treatment of an acute attack 'Daraprim' should be supplemented with a fast-acting schizontocide.

safe • *economical* • *tasteless*

Full information will be sent on request.

Tablet of 25 mg.
Supplied in boxes of 30 and bottles of 1,000.

BURROUGHS WELLCOME & CO. (U.S.A.) INC., Tuckahoe, New York

PERSISTENCE OF BACTERIA IN THE DEVELOPMENTAL STAGES OF THE HOUSEFLY

III. Quantitative Distribution in Prepupae and Pupae.*†

BERNARD GREENBERG

University of Illinois, College of Pharmacy, Chicago 12, Illinois

INTRODUCTION

A quantitative study of the normal flora in the larva, prepupa, pupa and emerging adult housefly discloses two significant declines manifest in the prepupa and newly emerged adult of laboratory-bred flies and of those breeding naturally in manure, privy contents and garbage.[6] Wollman[2] administered typhoid and dysentery organisms to aseptically reared housefly larvae but was unable to recover these organisms from adults. This was accepted by Duncan[3] as evidence for the presence of a bactericidal substance in the gut of the larva and pupa. His homogenate of the digestive tract, however, had no effect on typhoid or dysentery organisms, nor on *Escherichia coli* and *Pseudomonas* sp. Stewart[4] concluded, on the basis of histological evidence, that most, if not all, of the bacteria ingested by *Lucilia sericata* (Meig.) larvae were killed by the acid of the midgut. On the other hand, prepupae of another species of blowfly, *Calliphora erythrocephala* Hutton, retain 10^5 bacteria internally.[5] The housefly maggot, too, has a comparable portion of acid midgut, nevertheless we have also recovered large numbers of bacteria, mainly coliforms, *Proteus* and *Pseudomonas*, from its interior. Our present purpose is to suggest a mechanism to explain the reduction of bacteria in the prepupa and to describe the quantitative distribution of bacteria in the prepupa and pupa.

MATERIALS AND METHODS

Fly stages were reared in a normally contaminated larval medium (CSMA), employing the same general techniques for disinfecting, homogenizing and plating our specimens as previously described.[1, 6] Each specimen, whether midgut, hindgut, pupa case, etc., was diluted to 1 ml after grinding, from which further dilutions were made for plating. All materials used in the bacteriological procedures, *e.g.*, capillary pipettes, dissecting instruments, saline, etc., were sterile. Dissections were performed in well-slides, using micro-instruments fashioned from fragments of razor blades inserted into Pyrex glass rods. For dissecting needles, we used insect pins of various sizes, including *minuten nageln*, which were also inserted into glass rods. The instruments were rendered sterile by flaming, and the stereomicroscope's stage and other parts were swabbed with disinfectant. With normal precautions we found aerial contamination to be negligible. A detailed account of each procedure is given in the descriptions of the experiments which follow.

EXPERIMENTAL

Loss of bacteria in the prepupa. At the height of its growth, the maggot migrates from the moist, fermenting portions to the dry, crusted upper strata of the larval medium.‡ Here it wanders for about 24 hours, presumably without feeding. Dissection of the prepupa reveals an empty, contracted gut, quite unlike the gut of a feeding larva which is distended and filled with dark fluid. On the basis of these observations, we conjectured that the reduction of bacteria in this stage is related to a cessation of feeding. To test this hypothesis, we starved groups of feeding third instars kept in Petri dishes at 75 or 100% relative humidity (RH) and 33° C. After 24 hours, the maggots were disinfected and tested as usual. At this time, controls were taken from the larval medium and identically processed. The results show that at the end of a 1-day fast, the maggots have lost most of their flora, final

* This study was supported by a grant from the University of Illinois Research Board.

† The author gratefully acknowledges the contribution of Dr. Allen M. Burkman of the College of Pharmacy in preparing the statistical analysis of the data. He also wishes to thank Miss Esther Cooksey of Chicago for the preparation of the chart.

‡ This, at least, is the behavior of prepupae in the laboratory. Under natural conditions, they often migrate down through the manure to the cooler, damper soil below.

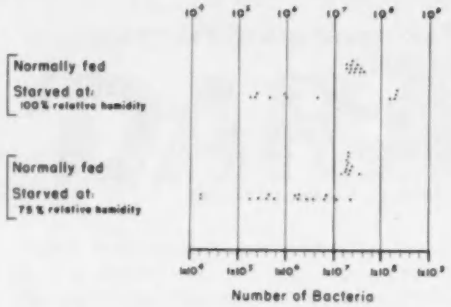


FIG. 1. Effect of starvation for 24 hours on retention of bacteria in third instar maggots.

TABLE 1

Bacteria in the hemolymph of mature maggots and prepupae

Sample	No. in sample	No. of specimens containing:			
		0 bacteria	<10 bacteria	<100 bacteria	<500 bacteria
Mature maggots	38	23	11*	2	1
Prepupae	48	44	4	0	0

* Majority with less than three bacteria.

counts being within a range similar to the range of counts typically observed in prepupae. The counts of control maggots had the usual cluster at 10^7 (Fig. 1).

Hemolymph of prepupae. The hemolymph of mature maggots and prepupae was readily obtained by puncturing the dorsal aorta of a disinfected specimen. The straw-colored fluid was collected in a capillary tube, expelled into a Petri plate, and mixed with melted agar. The results leave little doubt that the hemolymph of most maggots and prepupae is sterile (Table 1). It is therefore safe to assume that the larva's gut is an effective barrier to routine systemic invasion by the microorganisms inhabiting it.

Digestive tract of prepupae. Prepupae, rather than feeding third-instar maggots, were used for dissection of the digestive tract. The gut of the maggot, turgid with its fluid contents, ruptures easily and pours out contamination. The gut of the prepupa is empty, relatively firm and contracted, and therefore easily manipulated. It was removed in the following way. The extreme anterior and posterior ends of the specimen were snipped transversely and, with a flat blade, the gut was squeezed out posteriorly into a drop of saline. Adhering to the gut are clusters of fat cells and tracheae which were easily teased away. The gut was then transferred to another well-slide containing a drop of saline and the midgut was separated from the hindgut at the juncture of the Malpighian tubules. Each portion of gut was separately rinsed, ground and plated. The saline solutions for each step in the dissection were separately plated; these counts were so low as not to affect the final figures. Fat and tracheae were separately plated, with negative results.

The results of five series of such dissections show the hindgut to be generally more heavily contaminated than the midgut (Table 2).

Digestive tract of pupae. In dissecting pupae, we found the guts of those less than 36 hours old difficult to remove without gross internal contamination, for the contents of the early pupa are quite fluid due to the histolytic processes occurring during this time. We therefore used pupae, 2 days old or somewhat older, which were removed from the disinfected puparium in the following way. First, the anterior third of the puparium was cut away. The head was then impaled on a needle and the entire pupa gently withdrawn while the puparium was held with forceps. Enveloping the pupa is the transparent molting membrane. After peeling it away, the gut was pushed out posteriorly through a slit made in the last abdominal segment. The gut was then freed of adhering fat and tracheae, and processed in the same way as the prepupa. The results show that the guts of 2- and 3-day-old pupae retain relatively few bacteria and, in a few cases, are sterile (Table 2). Controls consisting of undissected pupae of similar age have typical counts near 10^5.

Other pupal structures. The marked disappearance of most of the bacteria from the pupal gut indicated a need for further study of the location and number of bacteria in the pupa. Using a sample of 3-day-old pupae, we performed the following experiments:

1. Puparia were disinfected and rinsed in the usual way.
2. The pupa was removed from the pupa case and the latter was ground and plated.
3. The pupa was placed on a well-slide and the molting membrane was removed as a sac by slitting it anteriorly and peeling it

TABLE 2

*Number of bacteria in the dissected midgut and hindgut of prepupae and pupae. The total count for a prepupa is the sum of the mid- and hindgut counts, times a dilution factor of 2. The counts are adjusted for direct comparison with those from undissected prepupae in this and the previous study.**

Stage and experiment number	Midgut	Hindgut	Total
Prepupae I	3.2×10^3	6.7×10^2	2.0×10^4
	1.9×10^4	1.4×10^4	6.6×10^4
	1.0×10^3	3.1×10^4	6.4×10^4
	1.2×10^3	7.0×10^2	3.8×10^3
	1.0×10^2	2.0×10^2	6.0×10^2
	4.2×10^3	1.4×10^4	3.6×10^4
Prepupae II	1.4×10^2	9.3×10^5	1.9×10^6
	7.8×10^2	1.0×10^6	2.0×10^6
	2.6×10^2	1.0×10^6	2.0×10^6
	3.6×10^2	2.4×10^4	4.8×10^4
	1.5×10^3	1.0×10^6	2.0×10^6
	8.2×10^3	1.0×10^6	2.0×10^6
Prepupae III	1.3×10^3	7.4×10^6	1.4×10^7
	1.0×10^1	3.0×10^2	6.2×10^2
	3.5×10^5	1.1×10^7	2.3×10^7
	4.2×10^5	1.6×10^5	1.2×10^6
	4.5×10^3	2.8×10^4	5.7×10^4
	1.0×10^6	1.5×10^7	3.2×10^7
	8.3×10^3	1.5×10^6	3.0×10^6
Prepupae IV	5.6×10^2	6.7×10^5	1.3×10^6
	8.7×10^3	9.0×10^4	2.0×10^4
	2.7×10^3	1.4×10^6	2.8×10^6
	6.1×10^3	4.3×10^5	8.6×10^5
	3.8×10^1	7.2×10^4	1.4×10^5
	4.7×10^2	5.0×10^5	1.0×10^6
	1.8×10^3	6.7×10^5	1.3×10^6
Prepupae V	5.2×10^3	4.2×10^4	9.4×10^4
	4.5×10^2	1.2×10^3	3.3×10^3
	3.2×10^4	2.3×10^4	1.1×10^5
	1.1×10^5	9.0×10^4	4.0×10^5
	1.2×10^3	1.2×10^3	4.8×10^3
	3.2×10^3	4.0×10^3	1.4×10^4
48-hr. pupae VI	0	3.0×10^1	3.0×10^1
	8.0×10^1	—	8.0×10^1
	4.0×10^1	—	4.0×10^1
	4.0×10^1	1.0×10^2	1.4×10^2
	2	—	2
	5	—	5
	3	—	3
	4	—	4
	2	—	2
	1	1	2
	3	—	3
72-hr. pupae VI	0	0	0
	0	0	0
	1	5	6

TABLE 3

Summary of data on the location and number of bacteria in the 3-day pupa

	Range	n	Mean	Standard error
Pupa case	4.0×10^2 to 2.2×10^6	12	7.4×10^5	1.4×10^5
Moulting membrane	5.0×10^2 to 5.3×10^6	11	6.3×10^5	4.5×10^5
Surface of pupa	1.0×10^2 to 4.8×10^4	12	1.3×10^4	4.5×10^3
Pupa minus gut	2.0×10^1 to 6.3×10^6	13	1.1×10^4	4.5×10^3
Midgut and hindgut	0 to 3.5×10^3	13	2.8×10^2	4.2×10^2

TABLE 4

Summary of bacterial counts of newly emerged flies and their puparia

	Range	n	Mean	Standard error
Fly	0 to 8.7×10^3	23	4.9×10^2	3.3×10^2
Puparium	2.1×10^4 to 1.8×10^6	23	3.8×10^5	1.0×10^5

back from the thorax and abdomen. The sac was ground and plated.

4. The pupa was washed in 10 ml of cold, 0.9% saline solution for 10 minutes; 1 ml of the wash water was plated.
5. The pupal gut was then removed and ground. In most cases, all of the hindgut and portions of the midgut and Malpighian tubes were obtained. At this stage, the posterior portion of the midgut is contracted and amber-green.
6. The remainder of the pupa was ground and plated. Fluid, fat and tracheae accumulated in the removal of the gut were plated, and the counts thus obtained were included in the pupal count.

* Student's t-test reveals no significant difference ($P > 0.05$) between mid- and hindgut counts in samples I and V. Differences between mid- and hindgut counts were highly significant ($P < 0.001$) for samples II, III, and IV.

The results indicate that the inner surface of the puparium becomes the repository for the largest number of bacteria. In declining order are the molting membrane, the surface of the pupa, the pupa with gut removed, and the gut itself. It must be emphasized that the juxtaposition of these various structures makes it impossible to remove them without cross-contamination. For this reason, it is perhaps preferable to interpret the data as indicating the relative numbers of bacteria associated with each structure (Table 3).

Newly emerged flies and their puparia. Disinfected pupae were kept singly in Petri dishes at room temperature and 100% RH till emergence. Flies, less than 30 minutes old, were immobilized by cold, ground and plated. Their empty puparia were also ground and plated (Table 4).

DISCUSSION

Mature, actively feeding maggots, when removed from the breeding medium and starved for a day, usually lose more than 90% of their bacteria. This reduction is of the same magnitude as the losses sustained by the nonfeeding prepupa. This may be indicative of bactericidal activity or the consequence of gut motility which aids in flushing out the bacteria. What supports the latter idea is the fact that fairly rapid changes occur in the gut after the maggot ceases to feed. Within 24 hours, the distended lumen shrinks to a fraction of its former caliber as the bacteria-laden fluid it contains is either absorbed or evacuated. Leach[7] sectioned prepupa of *Hylemya cilicrura* (Rond.), the seed-corn maggot, and found aggregates of bacteria in the folds of the gut wall, while the lumen was clear. It seems likely that the primary causes for bacterial decimation in the housefly prepupa are mechanical ones: curtailed feeding and continued elimination. These processes in the housefly prepupa reduce its count to 10^5 whereas in the hornfly, *Siphona irritans* (L.), autosterilization may actually be carried to completion.[8] These authors suggest that the causes for qualitative and quantitative reductions in the prepupal flora are bacterial antagonism, and digestion and elimination, respectively. They agree with Glaser[9] that the hornfly pupa and adult retain very few bacteria. The situation in this species departs from the typical picture for muscoid flies and merits further study of the factors contributing to sterilization of the gut.

The similar counts of prepupae and pupa cases suggest a relocation of the prepupa's intestinal flora. During the first day of pupation (at 35° C), the shedding of the cuticular lining of the fore- and hindgut displaces the majority of the bacteria from within the pupa to the inner surface of the pupa case, the molting membrane, and the surface of the pupa itself. During the remainder of the pupation period, these bacteria are able to maintain their numbers, situated in a relatively neutral environment, beyond phagocytic and other inimical host factors. In fact, they persist undiminished within the empty pupa case at least 2 days after eclosion, when kept at 100% RH. After the molt, the number of bacteria remaining in the mid- and hindguts of pupae is indeed small, but the sterilization of the midgut cannot be explained by the molting process. Perhaps, as others have suggested, it is a result of phagocytosis following midgut histolysis.

It may be worth comparing various findings on the number and location of bacteria in the pupa case. Leach[7] demonstrated in histological sections of *Hylemya cilicrura* pupae that bacteria pile up in the stomodeal and proctodeal regions following the ecdysis of the fore- and hindgut lining. Disinfected puparia from which adult *C. erythrocephala* have emerged yield only a few dozen bacteria,[5] whereas we have consistently found the housefly puparium heavily contaminated (Tables 3, 4). By dividing the puparium into three sections, we found each approximately equally contaminated. The small recovery reported by Balzam could result from losses during the longer pupation period*, or from the presence of few organisms in the hindgut of the pre-pupa. Among several points of agreement between Balzam's work and ours, two are noteworthy: the number of bacteria in the intact pupa remains relatively unchanged during pupation; and the pupal gut yields relatively few bacteria.

Of considerable epidemiological significance is the eclosion of the fly with a count near 10^2 from a puparium containing 10^5 bacteria. We could find no correlation between the two counts. Flies with very few bacteria may emerge from puparia with high counts. Seemingly, a large proportion of flies do not acquire contamination from the interior of the pupa case during eclosion. This is at once intriguing and difficult to explain.

* Balzam reports it as 12 days in this species of blowfly.

SUMMARY

1. Starvation experiments support the conclusion that the more than 90% decline in the number of bacteria in prepupae, as compared with mature maggots, is the result of a cessation of feeding and of continued elimination.

2. There is little likelihood of routine systemic invasion by the intestinal flora since, in the majority of maggots and prepupae, the hemolymph is sterile. Fat and tracheae are likewise sterile.

3. The hindgut of the prepupa typically harbors more bacteria than the midgut.

4. Dissections of pupae provide evidence for the translocation of the intestinal flora of the prepupa to the inner surface of the puparium, the molting membrane, and the surface of the pupa. The pupal gut retains very few organisms.

5. The fly emerges with a count near 10^2 from a puparium containing about 10^5 bacteria.

REFERENCES

1. Greenberg, B., 1959. Persistence of bacteria in the developmental stages of the housefly. I. Survival of enteric pathogens in the normal and aseptically reared host. *Am. J. Trop. Med. & Hyg.*, **8:** 405–411.

2. Wollman, E., 1921. Le rôle des mouches dans le transport de germes pathogènes étudié par la technique des élevages aseptiques. *Compt. Rend. Hebdom. Séances Acad. Sci. Paris*, **172:** 298–301.

3. Duncan, J. T., 1926. On a bactericidal principle present in the alimentary canal of insects and arachnids. *Parasitology*, **18:** 238–252.

4. Stewart, M. A., 1934. The role of *Lucilia sericata* (Meig.) larvae in osteomyelitis wounds. *Ann. Trop. Med. Parasitol.*, **28:** 445–460.

5. Balzam, N., 1937. Destin de la flore bactérienne pendant la métamorphose de la mouche a viande (*Calliphora erythrocephala*). *Ann. Inst. Pasteur*, **58:** 181–211.

6. Greenberg, B., 1959. Persistence of bacteria in the developmental stages of the housefly. II. Quantitative study of the host-contaminant relationship in flies breeding under natural conditions. *Am. J. Trop. Med. & Hyg.*, **8:** 412–416.

7. Leach, J. G., 1933. The method of survival of bacteria in the puparia of the seed-corn maggot (*Hylemia cilicrura* Rond.). *Z. Angew. Entomol.*, **20:** 150–161.

8. Stirrat, J. H., McLintock, J., Schwindt, G. W. and Depner, K. R., 1955. Bacteria associated with wild and laboratory-reared horn flies, *Siphona irritans* (L.). *J. Parasitol.*, **41:** 398–406.

9. Glaser, R. W., 1923. The survival of bacteria in the pupal and adult stages of flies. *Am. J. Hyg.*, **3:** 469–480.

PERSISTENCE OF BACTERIA IN THE DEVELOPMENTAL STAGES*† OF THE HOUSEFLY

IV. Infectivity of the Newly Emerged Adult

BERNARD GREENBERG

University of Illinois, College of Pharmacy, Chicago 12, Illinois

INTRODUCTION

The first and second papers of this series[1, 2] related the survival of *Shigella* and *Salmonella* in the larva, prepupa, pupa and adult housefly to the ecology of the larval medium. By studying the course of infection in normal and axenically-reared flies, we obtained evidence that the host's internal environment favors neither saprophytes nor pathogens. Both types suffer a comparable decline in numbers due to such nonspecific causes as cessation of feeding in the prepupa stage, and extrusion of the bacteria-laden intestinal lining, early in pupation. We attributed the sporadic and generally poor recovery of certain enteric pathogens from the various fly stages to suppressive overgrowth by the indigenous flora of the larval medium. Some of these species were isolated and identified. In the third paper[3], we presented data on the quantitative distribution of these bacteria in prepupa and pupa. The present paper extends this study to a bacteriological examination of the teneral adult.

Ledingham[4] tested the dissected gut of flies which emerged from sterilized pupa cases and found three of nine sterile. Of the positives, one had a single colony, while the others had moderate to heavy growth. Glaser[5] recovered large numbers of bacteria from the digestive tract of a small sample of newly emerged houseflies, but found none in *Stomoxys calcitrans*, the stable fly, and *Siphona irritans*, the horn fly. A progressive decline was observed in the number of bacteria within the pupae of unidentified species of *Sarcophaga* and *Sarcophagula*[6]; few or no bacteria were recovered from surface washings of newly emerged adults, nor from the interior of adults 2 days after eclosion. At emergence, the majority of blowflies, *Calliphora erythrocephala*, contain more than 1,000 bacteria, but 1 day later, are sterile.[7] Finding relatively few organisms in the pupal gut or puparium, Balzam hypothesized the systemic invasion of the intestinal flora early in pupation, their residence in the tissues, and phagocytic destruction during the first day after eclosion. Bacot[8] studied *Pseudomonas aeruginosa* in the housefly and concluded that the pupal gut is heavily infected. This, however, was based on the questionable technique of crushing the entire pupa.[3] He further stated that in the imago, infection is maximal at emergence and then diminishes suddenly.

Perhaps it is apparent, even from this brief review, that available knowledge on the subject, particularly with regard to the housefly, is fragmentary and unsettled. For this reason, and because of its epidemiological implications, we used several approaches in an effort to collect adequate data bearing on the infectivity of the adult's gut at emergence. We performed the following experiments with teneral adults:

1. Testing of feces and meconium of flies reared with a single species of human pathogen or with a multiple saprophytic flora;
2. Cumulative sampling of feces from flies maintained aseptically for 2 days;
3. Testing of digestive tract;
4. Sampling of bacteria on the surface and within the fly.

MATERIALS AND METHODS

Housefly larvae were reared in the routine manner in a naturally contaminated breeding medium (CSMA). Pupae were disinfected and kept singly in 60-mm Petri dishes at room temperature (ca. 25° C) and 100% relative humidity (RH). Newly emerged adults, no older than 30 minutes, were immobilized by cold to facilitate experimental manipulations. Descriptions of the sterilization, disinfection, grinding and other

* This study was supported by a grant from the University of Illinois Research Board.

† The author gratefully acknowledges the contribution of Dr. Allen M. Burkman of the College of Pharmacy in preparing the statistical analysis of the data. He also wishes to thank Miss Esther Cooksey of Chicago for the preparation of the illustrations.

techniques, and the bacteriological procedure, are found in the preceding papers of this series.[1, 2, 3] All materials used in bacteriological procedures, *e.g.*, rinse water, saline and sucrose solutions, grinding tubes, vials, dissecting instruments, etc., were sterile. A detailed protocol is given in the description of each of the experiments which follows.

EXPERIMENTAL

Feces and meconium. In this study, we compared flies reared normally, in a larval medium containing a multiple flora, with others reared in a medium containing a single species of pathogen.

TABLE 1

Infectivity of meconium and feces of newly emerged flies reared normally or with single species of bacteria

Species of bacteria	Flies tested	Flies containing:				
		0 bacteria	< 10 bacteria	< 100 bacteria	< 1000 bacteria	< 10,000 bacteria
Shigella flexneri	13	13	0	0	0	0
Salmonella enteritidis	10	9	1	0	0	0
Salmonella typhi	15	4	6	1	2	2
Normal flora	14	11	3	0	0	0

For the latter, we used *Shigella flexneri*, *Salmonella enteritidis* and *S. typhi*. Whitish-green urates which begin to accumulate in the rectum of the 3-day old pupa are usually voided by the fly shortly after emergence. In addition, a few drops of clear, liquid feces may also be passed. With the fly immobilized, these wastes were collected in capillary pipettes and separately plated. The absence of any significant difference permits incorporating the results for the feces and meconium into one table. These are generally sterile or have less than 10 bacteria. In a few i-n stances, the counts are as high as 10^2 (Table 1).

Cumulative sampling of feces. The collection and testing of feces from flies maintained aseptically for 2 days after emergence was accomplished in the following manner. Small paraffin blocks were sterilized by immersion in aqueous $HgCl_2$ (1:500) for 24 hours, and then thoroughly rinsed. Flies immobilized by cold were fixed to the block by melting paraffin around the wings. The block, in turn, was fixed to the inside of a 5 × 2-cm shell vial by applying a red-hot needle to the outside of the glass until the paraffin melted against it.[9] The final position of the fly was vertical, its abdomen directed downward, extending beyond the paraffin block (Fig. 1). One ml of saline was added to the vial which was replugged and placed in a humidity chamber at room temperature (100% RH; ca. 25° C). By means of a bacteriological loop, flies were fed a 5% sucrose solution 1 and 2 days after emer-

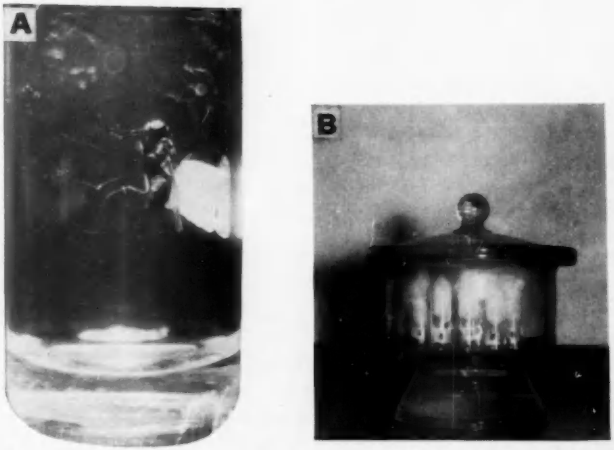


FIG. 1. a. Newly emerged fly mounted over sterile saline solution. b. Same in humidity chamber

gence. Flies were not inclined to feed during the first few hours after emergence, but readily imbibed the proffered solution after 1 day of fast. Since feeding stimulates defecation, we fed the flies shortly before testing the saline, to improve the recovery of bacteria. At the end of the 1st day, paraffin block and fly were transferred as a unit to another vial into which 1 ml of saline was pipetted. The 1st day's saline was then plated. After the 2nd day, the fly was removed from the block, ground and plated without prior washing. The 2nd day's saline was also plated. We thus obtained data on the infectivity of the feces during the first 2 days of adult life. The recovery of bacteria during the 1st day was slight, with the majority of samples between 0 and 10. The 2nd day's sampling exhibited no change. The flies themselves had counts ranging from 0 to 10^3, with the majority having less than 100 bacteria (Table 2). Controls from the population cage were at least 3 days old and had been feeding on the regular milk and sugar solution in a normally contaminated environment. They were similarly treated and gave counts of 4.5×10^3, 7.2×10^3 and 5.1×10^4 for the 1st day's saline sampling, and 4.0×10^3, 3.2×10^3 and 7.1×10^3, respectively, for the 2nd day's saline.

TABLE 2

Summary of data on the infectivity of fly feces collected from newly emerged adults maintained aseptically for 48 hours

Counts	Bacteria in feces after		Bacterial count of fly at end of 2 days
	1st day	2nd day	
Range	0–8.2×10^1	0–4.4×10^1	0–5.8×10^2
n	18	17	18
Mean	6.2	6.0	7.7×10^2
Standard error	4.5	2.8	4.2×10^2

Dissected digestive tract. In the first such experiment, teneral adults were disinfected in White's solution and rinsed six times before they were dissected. We decided to eliminate the disinfecting procedure because the agar plates remained sterile, on the chance that the disinfectant had penetrated into the gut and destroyed the organisms. Flies, thereafter, were neither disinfected nor washed. Specimens were held by the thorax with a broad-tipped forceps while the end of the abdomen was gripped with a fine-tipped forceps and the digestive tract was gently withdrawn into a drop of saline. By this means, we usually obtained all of the hindgut and almost all of the midgut, Malpighian tubes included, but the foregut was not recovered. These structures were ground as one and plated. For controls, we processed the digestive tracts of disinfected flies taken from the population cage. Their counts were 5.0×10^3, 2.0×10^4, 6.2×10^4, and 1.1×10^5. The digestive tracts of 20 test flies were sterile, save one with a count of eight, and another with one colony.

Bacteria on the exterior and interior. The object of this experiment was to explore further the general distribution of bacteria in the teneral adult. Flies, emerging from disinfected puparia into a sterile container, were treated in one of several ways:

1. Flies were disinfected externally by immersion and vigorous agitation for 10 minutes in

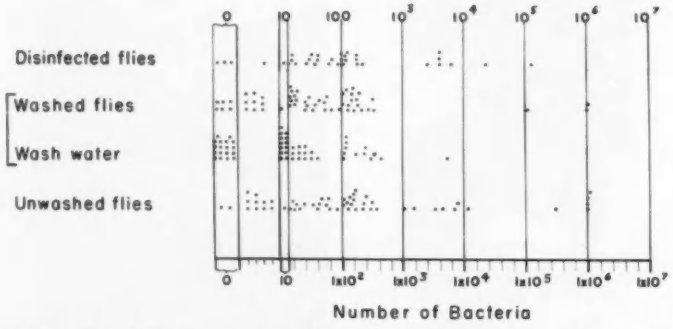


FIG. 2. Effect of surface disinfection and washing on the numbers of bacteria retained by the newly emerged adult. Each dot represents a single sample or specimen.

White's solution. The disinfected specimens were then rinsed six times, transferred to thioglycollate broth for an additional 10 minutes, removed, ground, and plated. If growth appeared in the broth after 72 hours at 35° C, the datum for the specimen was discarded.

2. Flies were individually and vigorously agitated for 10 minutes in 10 ml of cold, distilled water containing 0.01% aerosol. The specimen was then removed, ground, and plated, and a sample of the wash water was also plated.
3. Flies were ground and plated without prior treatment.

The number of bacteria recovered from the surface of a fly was generally less than 50, with a somewhat larger number retained internally (Fig. 2). The surface counts must not be interpreted too literally, because many organisms may have adhered to the fly.

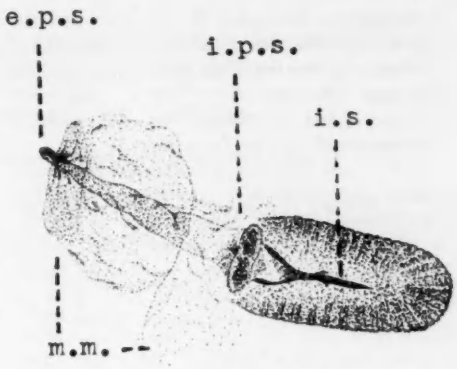


FIG. 3. Respiratory structures of 3-day-old pupa. Note pupal tracheal tube entering imaginal spiracle (i.s.). The tube has two spiracles—an internal pupal spiracle (i.p.s.) and an external pupal spiracle (e.p.s.)—which open under the molting membrane (m.m.) and outside the pupa, respectively.

DISCUSSION

Tests of the meconium, feces and dissected intestinal tracts of teneral adults, and cumulative sampling of their feces indicate that the fly's gut contains few if any bacteria. This information, coupled with our previous findings, suggests a general process of gut sterilization which commences before pupation and is completed by the time of eclosion.[3] The extraintestinal flora persists without marked increase or decrease for at least the first 2 days of the adult's life. The number of surface contaminants acquired naturally is probably greater than in the laboratory, and would depend on the nature and height of the material through which the fly burrows to reach the surface. We have not yet tried to locate the organisms which persist internally, but suspect their presence in the thoracic tracheae. The molt which ejects the intestinal flora during the 1st day of pupation discloses a rather interesting arrangement of pupal and adult respiratory structures (Fig. 3). The prothoracic spiracle of the adult is penetrated by the pupal trachea which opens on the outside of the pupa case and also on the surface of the pupa. Perhaps sufficient moisture exists to permit the passage of bacteria through the prothoracic spiracles into the shelter of the tracheal tubes and sacs within.

Typical associates of newly emerged houseflies are *Proteus*, *Pseudomonas* and various coliforms.[2, 10, 11] There is justification for categorizing them as potential human pathogens because of their increasing implication in diarrheal diseases, especially of infants. Certain of the true pathogens, *e.g.*, *S. enteritidis* and *S. paratyphi* B, may also be present in the adult, but their numbers would probably be quite low, considering the relatively low counts of most newly emerged flies. The studies of McCullough and Eisele[12] on experimental human salmonellosis indicate the magnitude of the dose necessary to produce symptoms (minimum 10^5, average about 10^6 or 10^7 organisms). On the basis of dosage delivery, the risk of human infection from such a fly is probably slight.[13] On the other hand, *S. pullorum* may be effectively established among chickens by ingestion of infected flies,[14] and perhaps prepupae, which are subject to predation by various animals while migrating from infected breeding material, may also play a part. Another possible means of infection in animals is suggested by the recovery of viable *Salmonella* in flies 7 days after they had been killed with DDT.[15] Though *Salmonella* and *Shigella* ingested by larvae apparently have no effect on the longevity of the adult, such is not the case with *Pasteurella pestis*. Gosio[16] infected housefly and blowfly larvae and found that, though the count fell in the pupa, there was a bacteremia in the adult followed by death within 24 hours of emergence. Bacteremia in muscoid flies infected with human pathogens is an unusual phenomenon and it should be emphasized that successful transmission

of pathogens is more often the exception than the rule when normal contamination of the larval medium is permitted. This has been shown for polio and other viruses;[17, 18] for *S. pullorum*;[19] for *S. typhi* and *Sh. flexneri*;[1] for several species of *Leptospira*;[20] and for *Toxoplasma*.[21]

This is not intended to minimize the transmission potential of the fly once it takes wing. The number and kinds of microorganisms it acquires in its lifetime will depend on the availability of excrement and other wastes in its home range. A single visit to such material could serve to recolonize its gut and contaminate its exterior.

SUMMARY

An evaluation of the infectivity of the newly emerged adult housefly is based on a quantitative bacteriological study of the following:

1. Feces and meconium of flies reared with a single species of human pathogen or with a multiple saprophytic flora;
2. Cumulative sampling of feces from flies maintained aseptically for 2 days;
3. Testing of digestive tract;
4. Sampling of bacteria on the surface and within the fly.

Data for the first three categories indicate that the fly's gut contains very few bacteria. This is further evidence for a general process of gut sterilization which commences before pupation and is completed by the time of eclosion. The data for the fourth category show that surface contaminants account for only a part of the remaining flora, the rest of which is retained internally at counts of about 100. The epidemiological implications of these findings are discussed.

REFERENCES

1. Greenberg, B., 1959. The persistence of bacteria in the developmental stages of the housefly. I. Survival of enteric pathogens in the normal and aseptically reared host. *Am. J. Trop. Med. & Hyg.*, **8:** 405–411.
2. Greenberg, B., 1959. The persistence of bacteria in the developmental stages of the housefly. II. Quantitative study of the host-contaminant relationship in flies breeding under natural conditions. *Am. J. Trop. Med. & Hyg.*, **8:** 412–416.
3. Greenberg, B., 1959. The persistence of bacteria in the developmental stages of the housefly. III. Quantitative distribution in pre-pupae and pupae. *Am. J. Trop. Med. & Hyg.*, **8:** 613–617.
4. Ledingham, J. C. G., 1911. On the survival of specific microorganisms in pupae and imagines of *Musca domestica* raised from experimentally infected larvae. Experiments with *B. typhosus*. *J. Hyg.*, **11:** 333–340.
5. Glaser, R. W., 1923. The survival of bacteria in the pupal and adult stages of flies. *Am. J. Hyg.*, **3:** 469–480.
6. Nicholls, L., 1912. The transmission of pathogenic microorganisms by flies in Saint Lucia. *Bull. Ent. Res.*, **3:** 81–88.
7. Balzam, N., 1937. Destin de la flore bactérienne pendant la métamorphose de la mouche a viande (*Calliphora erythrocephala*). *Ann. Inst. Pasteur*, **58:** 181–211.
8. Bacot, A. W., 1911. The persistence of *Bacillus pyocyaneus* in pupae and imagines of *Musca domestica* raised from larvae experimentally infected with the bacillus. *Parasitol.*, **4:** 68–73.
9. Frings, H., 1946. The mounting of living insects for observation and study. *Turtox News*, **24:** 150–154.
10. Steinhaus, E. A., 1946. *Insect microbiology*. Comstock Publ. Co., Inc., Ithaca, New York, pp. 120–132.
11. West, L. S., 1951. *The housefly*. Comstock Publ. Co., Inc., Ithaca, New York, ch. 10, 11.
12. McCullough, N. B., and Eisele, C. W., 1951. Experimental human Salmonellosis. IV. Pathogenicity of strains of *Salmonella pullorum* obtained from spray-dried whole egg. *J. Inf. Dis.*, **89:** 259–265; (cites three prior studies).
13. Lindsay, D. R., and Scudder, H. I., 1956. Nonbiting flies and disease. *Ann. Rev. Entomol.*, **1:** 323–346.
14. Gerberich, J. B., 1952. The housefly (*Musca domestica* Linn.) as a vector of *Salmonella pullorum* (Rettger) Bergey, the agent of white diarrhea of chickens. *Ohio J. Sci.*, **52:** 287–290.
15. Gross, H., and Preuss, U., 1951. Infektionsversuche an Fliegen mit darmpathogenen Keimen. Versuche mit Typhus und Paratyphusbakterien. *Zentralbl. Bakt.*, Abt. I Orig., **156:** 371–377.
16. Gosio, B., 1925. Uber die Verbreitung der Bubonenpesterreger durch Insektenlarven. *Arch. Schiffs. u. Trop. Hyg.*, **29** (Beiheft 1)**:** 134–139.
17. Bang, F. B., and Glaser, R. W., 1943. The persistence of poliomyelitis virus in flies. *Am. J. Hyg.*, **37:** 320–324.
18. Melnick, J. L., and Penner, L. R., 1952. The survival of poliomyelitis and Coxsackie viruses following their ingestion by flies. *J. Exp. Med.*, **96:** 255–271.
19. Gwatkin, R., and Dzenis, L., 1952. Studies in pullorum disease. XXIX. Bacteriological examination of blowflies (*Lucilia* sp.) and houseflies (*Musca domestica*) which during their larval stage had fed on chicks infected with *Salmonella pullorum*. *Canad. J. Comp. Med. Vet. Sci.*, **16:** 148–150.
20. Kunert, H., and Schmidtke, L., 1952. Die Bedeutung der nichtstechenden Fliegen fur die Verschleppung von Leptospiren. *Zeitschr. Tropenmed. u. Parasitol.*, **3:** 475–486.
21. Schmidtke, L., 1955. Histologische Untersuchungen an toxoplasmainfizierten Insekten (*Calliphora erythrocephala*, *Periplaneta americana*). *Zentralb. Bakt.* Abt. I Orig., **164:** 508–513.

CHLOROQUINE COMPARED WITH CHLOROQUINE AND EMETINE COMBINED IN AMEBIC LIVER ABSCESS

A. J. WILMOT, S. J. POWELL AND E. B. ADAMS

*Department of Medicine, University of Natal and the Amoebiasis Research Unit, Durban**

In amebic liver abscess we have shown that there is a higher relapse rate in patients treated with chloroquine alone than in those treated with 2 courses of emetine.[1] A further trial reported here has been undertaken to find out whether this relapse rate might be reduced by increasing the dose of chloroquine or by combining chloroquine with a single course of emetine.

MATERIAL AND METHODS

Diagnostic criteria and methods of selection were the same as in our previous report where they have been given in full.[1] Forty-eight consecutive male African patients with amebic liver abscess were allotted to one of two treatment groups by random selection. In all patients the diagnosis was established by the aspiration of pus from which *Entamoeba histolytica* was isolated in 40 cases (83%). Further aspirations were done when indicated. The treatment schedules were:

Group A. Emetine hydrochloride grains 1 intramuscularly daily for 10 days.
Chloroquine sulphate or diphosphate (dose expressed as mgm of the base) 600 mgm immediately, followed by 300 mgm 6 hours later and then 150 mgm twice daily for 29 days.
Diiodohydroxyquinoline 600 mgm thrice daily for 20 days.

Group B. Chloroquine sulphate or diphosphate (dose expressed as mgm of the base) 600 mgm immediately, followed by 300 mgm 6 hours later and then 150 mgm thrice daily for 29 days.
Diidohydroxyquinoline 600 mgm thrice daily for 20 days.
Tetracycline, 1 gram daily for 10 days, was given to patients with active amebic dysentery.

Diiodohydroxyquinoline and tetracycline are generally held to be inactive in hepatic amebiasis and were used only to control the intestinal parasites.

In both groups all drugs were started at the same time. Twenty-two patients were treated with emetine and chloroquine (Group A) and 23 with chloroquine (Group B). All patients were observed in hospital for 30 days and then instructed to return at intervals of 1, 3 and 6 months for assessment.

Three patients died. Two were in Group A. In one of these there was a massive rectal hemorrhage from active bowel amebiasis as well as rupture of the liver abscess into the peritoneum. The other died within 24 hours of admission and at autopsy numerous abscesses were found in both hepatic lobes. The death in Group B followed rupture of the abscess into the peritoneum. It is felt that these three cases do not influence the assessment of relative efficacy of therapy and they have consequently been excluded from the series.

RESULTS

The initial response of all patients was similar and satisfactory. There were no relapses in Group A compared with 5 (21%) in Group B. In three of these pus was again aspirated from the liver while hepatic pain and tenderness recurred in two. Toxic effects were noted only in Group B in which two patients vomited for a period of 2 to 3 days and one complained of nausea.

CONCLUSIONS

The significant relapse rate when chloroquine is given alone, even in high dosage, indicates that it is inadequate therapy for amebic liver abscess. It is known that a single 10 day course of emetine alone is also inadequate.[2] We have shown that 2 courses of emetine greatly reduce the relapse rate.[1] Our results now indicate that a single course of emetine combined with a standard dose of chloroquine (Group A) provides satisfactory cure with little tendency to relapse. This form

* The Amoebiasis Research Unit is sponsored by the following bodies: The South African Council for Scientific and Industrial Research, The Natal Provincial Administration, The University of Natal, The United States Public Health Service (Grant E/1592).

of treatment in adults is recommended as the most satisfactory method at present available.

It has two advantages over the schedule using 2 courses of emetine:

(a) Less emetine is used, reducing the risk of toxic effects on the heart; (b) A shorter period in hospital is usually possible, since patients who are progressing satisfactorily can be discharged at the end of their 10 day course of emetine, to complete chloroquine at home.

Chloroquine alone remains the drug of choice in patients in whom cardiovascular disease contraindicates emetine therapy. All forms of drug therapy should be supplemented by aspiration when necessary.

ACKNOWLEDGMENT

We wish to thank Dr. S. Disler, Medical Superintendent of King Edward VIII Hospital for facilities.

REFERENCES

1. Wilmot, A. J., Powell, S. J., and Adams, E. B., 1958. The comparative value of emetine and chloroquine in amebic liver abscess. *Am. J. Trop. Med. & Hyg.* **7**: 197.
2. Wilmot, A. J., 1949. Clinical manifestations of amoebiasis in the Bantu. A dissertation submitted for the degree of Doctor of Medicine, University of Oxford.

INVESTIGATION OF PARASITIC INFECTIONS IN THE CENTRAL AREA OF PHILADELPHIA

DANIEL WEINER*, M. M. BROOKE*, AND A. WITKOW†

Within the past 8 years, over 18,000 Puerto Ricans have taken up residence in Philadelphia, primarily in the central area of the city. The first awareness of a parasitic problem among these new residents arose through the activities of a health and welfare clinic (Casa del Carmen) sponsored by the Roman Catholic diocese. More recently, clinics of a limited nature have been operated by the city health department. In connection with this work, public health nurses have visited the homes of infected individuals to encourage them to obtain further medical attention. A survey of preschool Puerto Rican children has been made by medical students employed during the summer months.[9]

In order to obtain additional objective data on the parasitological situation in Philadelphia, an intestinal parasite survey of school children and a housing quality survey were conducted in the fall of 1957. The results of these surveys are summarized in the present paper.

METHODS AND RESULTS

School survey. The school survey was conducted in 2 public and 2 parochial schools attended by Puerto Rican and Negro and white children. (Table 1) Since racial classification of Puerto Rican children was not feasible, they were classified as an ethnic group. A large proportion of the Puerto Rican children attended one of the parochial schools and all of the Negroes attended one of the public schools. Only children in the fourth, fifth, and sixth grades (8 to 12 years of age) were included in order that they might be old enough to supply the information desired. All the children came from similar social and economic environments and resided in the central area of the city.

Health information on each child regarding headaches, abdominal pains, muscular pain, and upset stomach was obtained at the time stool collection kits were distributed. An affirmative notation was recorded if the child indicated that there was a history of any of these complaints 1 month prior to questioning. Height and weight were recorded and examinations were made for pallor, oral ulceration, potbelly, and paleness of the mucosa.

Stool specimens were submitted unpreserved, and in PVA-fixative[3] from 89% of the children interviewed. A single specimen was collected from each child. They were examined either in the city laboratory or in that of the state health department using primarily the formalin-ether concentration technique[11] and permanently stained PVA films. The parasitology laboratories of the Communicable Disease Center re-examined 75% of the specimens randomly selected.

School Survey Results. Table 2 presents the results of stool examinations according to the percentages of 385 children which were found to be infected with various species of parasites. It should be noted that the connotation "white" is used for white non-Puerto Rican children, and "Negro" is used for Negro non-Puerto Rican children. The results of stool examinations of the 10 Mongolian children are not included in the table. The stool examinations revealed 83.2% of the Puerto Rican, 30.2% of the white and 51.0% of the Negro children infected with one or more parasites. With regard to the protozoan infections, although the rates tended to be less in the white children there was no consistent pattern. The percentages for *Endolimax nana* and *Giardia lamblia* were highest among the Puerto Ricans, whereas *Dientamoeba fragilis* and *Trichomonas hominis* were highest among the Negro children. Practically all of the helminth infections were found in the Puerto Rican children. Specific examinations for *Enterobius vermicularis* were not performed, although a few infections were found on the routine stool examinations.

As a basis for comparison, an amebic prevalence rate (APR), and a helminth prevalence rate (HPR), were used. Table 3 presents a comparison

* Communicable Disease Center, Public Health Service, Department of Health, Education and Welfare, Atlanta, Georgia and Seattle, Washington.

† Formerly, Director, Division of Preventive Medicine, Philadelphia Department of Public Health, Philadelphia, Pennsylvania (Present address: Commissioner of Health, City Building, Hamilton, Ohio).

TABLE 1

Children in four schools examined for intestinal parasites

School	Total Number Examined	Groups			
		Puerto Rican (No.)	Non-Puerto Rican		
			White (No.)	Negro (No.)	Mongolian (No.)
Cathedral	106	106	0	0	0
St. Francis Xavier	120	1	119	0	0
Ludlow	55	55	0	0	0
Jefferson	114	5	50	49	10
Totals	395	167	169	49	10
Percentage of Totals	100	42.5	42.8	12.4	2.5

TABLE 2

Parasitological results according to groups of children examined

Results	Puerto Rican (167 children) %	Non-Puerto Rican		Total (385 children) %
		White (169 children) %	Negro (49 children) %	
Positive for Parasites	83.2	30.2	51.0	57.1
Protozoa identified:				
Entamoeba histolytica	6.6	3.0	8.2	5.2
Entamoeba coli	26.3	11.2	24.5	18.2
Endolimax nana	22.8	14.8	14.3	19.0
Iodamoeba butschlii	4.2	1.8	0	2.6
Dientamoeba fragilis	4.2	5.3	24.5	7.5
Giardia lamblia	20.4	7.7	8.2	13.8
Chilomastix mesnili	3.0	1.2	4.1	2.6
Trichomonas hominis	1.8	3.0	8.2	3.4
Helminths Identified:				
Trichuris trichiura	67.7	0	2.0	29.4
Hookworm	21.0	0	0	9.1
Ascaris lumbricoides	3.6	.6	0	1.9
Enterobius vermicularis	.6	2.4	2.0	1.9
Strongyloides stercoralis	.6	0	0	.3
Hymenolepis nana	1.8	0	0	.8
Schistosoma mansoni	3.0	0	0	1.3

TABLE 3

The amebic and helminth prevalence rates in groups of school children

Prevalence index	Puerto Rican (167 children) %	Non-Puerto Rican	
		White (169 children) %	Negro (49 children) %
Amebic (APR)	40.7	20.7	32.7
Helminth (HPR)	7.0	30.7	4.1

TABLE 4

Selected parasite rates in Puerto Rican children residing in the United States for different periods of time

Index or Organism	Months of residence in United States			
	0–24 Months (50 children) %	25–48 Months (46 children) %	49–72 Months (39 children) %	Over 72 Months (31 children) %
Amebic (APR)	32.0	54.0	38.5	38.7
Helminth (HPR)	68.0	78.0	82.1	48.4
Trichuris trichiura	68.0	71.7	79.5	45.2
Hookworm	28.0	30.4	17.9	0

between the groups of children studied with regard to both rates. The APR is based upon the number of individuals infected with *Entamoeba histolytica*, *Entamoeba coli* and/or *Endolimax nana*.[4] The rate is derived on an individual basis and demonstrates infection with one or more of these three parasites. The HPR is based on the number of individuals infected with one or more of the identified helminths with the exception of *Enterobius*. Although there are differences in the life cycles of the helminths, the HPR provides a composite index which can be used in the overall group comparisons. Both the APR and HPR were highest for the Puerto Rican children. The very high HPR for the Puerto Rican children in comparison with the other groups is due to the high prevalence rates of primarily *Trichuris trichiura* and hookworm.

An attempt was made to correlate length of residence in the United States with prevalence of infection of the Puerto Rican children. Table 4 presents prevalence data for various lengths of residence in the United States. The majority

(81.4%) of the Puerto Rican children had been in the United States for a period of six years or less. It should be noted that the APR remains rather constant for the time interval shown. On the other hand, the HPR drops significantly after six years of residence. This is paralleled by the rates for *T. trichiura* and hookworm. It is generally believed that infections of *Necatur americanus* are lost in four or five years. None of the 31 children residing in the United States for over six years was found infected with hookworm. The percentage of children that had been treated was low.

Table 5 presents a correlation of the percentages of infected and noninfected children with the subjective and objective signs recorded during the interviews. In all instances infected children demonstrated a higher occurrence of these signs, but it should be recognized that the various racial groups were not equally represented in the different categories.

Housing Survey. In order to gain further insight into the housing problems and facilities, the general housing conditions of Puerto Rican people in Health District 6 were surveyed. No attempt was made to evaluate all houses in the area. However, health examination records in the 6th Health District were studied and those containing cases of either amebiasis or hookworm were assigned to the sanitarian for field inspection. If one of these cases occurred in a multi-unit structure, all of the dwelling units in the structure were inspected. Procedures used in studying the physical conditions present in these homes were those developed by the American Public Health Association in its appraisal methods for measuring quality of housing.[3] It is widely recognized as a standard technique for providing comprehensive and objective information on housing inadequacies. The results of this survey were compared with the previous housing surveys conducted in Philadelphia using a comparable area (Hawthorne) and a superior area (Haddington) located outside of the central zone of the city.[5, 7]

TABLE 5

Subjective and objective signs in parasitized children and in those without demonstrated parasites

Objective and subjective signs	Children with parasitic infection (220)	Children with no parasitic infection (175)
	Per cent	Per cent
Pallor	42.3	29.1
Mucous membrane	59.1	45.7
"Pot belly"	22.3	2.9
Oral ulceration	2.7	1.7
Headache	34.1	13.7
Abdominal pain	25.5	16.6
Muscular pain	9.5	6.3
Upset stomach	26.4	11.4

TABLE 6

Comparison of major findings of housing survey health district 6 and other areas

Findings	Dwelling units studied in health district 6 (205 units)	Hawthorne area (1822 units)	Haddington area (405 units)
Owner occupied	12.7%	14.0%	59.0%
Facilities			
Poor	54.3	64	4
Substandard	35.6	17	14
Fair	10.3	19	82
Maintenance			
Poor	34.6	59	2
Substandard	40.0	28	14
Fair	25.4	13	84
Occupancy			
Overcrowded	38.1	7	5
Fair	62.1	93	95
Dwelling quality			
Poor	62.9	73	3
Substandard	28.3	23	33
Fair	8.9	4	64

Housing Survey Results. Table 6 presents a comparison of the housing quality in Health District 6 with those of the two other areas in Philadelphia. Two hundred and five dwelling units were completely studied in the 6th Health District area. Owner occupancy was low (12.7%). The majority were small to moderate in size, consisting of one to five rooms. These units were mainly occupied by Puerto Ricans and Negroes.

Conditions of these dwelling facilities demonstrated that both equipment and basic design are worse than marginal in over 50% of the dwellings. Important maintenance problems exist in over ⅓ of the units, while more than an equal number have substantial maintenance difficulties. Over ⅓ of these dwellings are seriously overcrowded. As a measure of the total dwelling quality (facilities, maintenance and occupancy) it was found that over 90% were extremely poor or substandard. In practically all categories, the housing conditions were somewhat better in Hawthorne and decidedly better in Haddington.

DISCUSSION

It is obvious that the prevalence of parasitic infection is much higher in Puerto Rican children than in non-Puerto Rican children living in Philadelphia. This is understandable since the prevalence rates for parasitic infections are high in Puerto Rico.[1, 7, 10, 12, 13, 8] Direct comparison with the results of the other surveys conducted in Puerto Rico cannot be made because of the differences in age groups involved, techniques employed and objectives sought.

The decrease in HPR of the Puerto Ricans after prolonged residence in the United States and the rarity of helminth infections in native children suggests that helminths are not being transmitted to any significant degree in Philadelphia. Owing to its northern location and urban environment, transmission of *Schistosoma mansoni*, *Strongyloides stercoralis*, and hookworm, with their complex life cycles, would not be expected to occur.

The fact that the APR for the Puerto Rican children remained on a rather constant level during the United States period of residence probably demonstrates that the protozoa are being transmitted in the area. This is also indicated by the relatively high APR in the non-Puerto Rican groups. The prevalence rates of protozoan infections in the Negro and white non-Puerto Rican children are almost as high in some instances as the rate in the Puerto Rican children, higher in others.

Protozoan infections are not limited to tropical climates and may be expected to be present wherever poor sanitation and hygiene exist. Of course this is likewise true for various other infectious diseases. This places great emphasis on the need for proper housing facilities and their maintenance. The examination of the housing facilities in the area shows them to be markedly inadequate in many aspects and decidedly inferior to the non-central areas of the city. The area studied is overcrowded and deficient in toilet and bathing facilities, which in all likelihood must facilitate transmission by direct contact.

SUMMARY

In order to obtain data on the parasitological situation in Philadelphia resulting from an increase in Puerto Rican residents an intestinal parasite survey of school children and a housing quality survey were conducted in the fall of 1957.

The children were from similar socio-economic environments and were attending the 4th, 5th, and 6th grades of 2 parochial and 2 public schools. Of 395 stool specimens examined 42.3% were from Puerto Rican children and 58.7% were from non-Puerto Rican children (white, Negro, or Mongolian). The examinations revealed 83.2% of the Puerto Rican, 51.0% of the Negro and 30.2% of the white children infected with parasites. A single stool specimen was examined from each child.

Fifteen different parasites were identified. The highest prevalence of any single species was for *Trichuris trichiura* (67.7% in the Puerto Rican children). Excluding *Enterobius vermicularis* for which specific examinations were not performed, the prevalence rates for all of the helminths were higher in the Puerto Rican children. Although generally true for the protozoa, several of the species had the highest prevalence in the non-Puerto Rican Negro group (e.g. 8.2% for *Entamoeba histolytica*). The prevalence of *Dientamoeba fragilis* (24.5%) for the Negro children was four to five times higher than for the other groups.

A significant reduction was observed in the helminth prevalence among the Puerto Rican children after six years of residence in the United States. However, there was not a similar reduction in amebic prevalence rate with length of residence. Furthermore, the protozoan infections were relatively high in the non-Puerto Rican children. These observations may demonstrate that although helminth infections (excluding *E. vermicularis*) are not being transmitted to any significant degree in Philadelphia, protozoan infections probably are. The housing quality survey revealed that the dwellings in the central area of Philadelphia are over-crowded and deficient in toilet and bathing facilities which in all likelihood must facilitate transmission by direct contact.

ACKNOWLEDGMENTS

Grateful appreciation is expressed to the following individuals without whose assistance this study could not have been accomplished: Dr. Carl C. Janowsky, Director, Division of Epidemiology, Philadelphia Department of Public Health (formerly District Health Director, Health District #6); Dr. Ruth Weaver, Director, Division of Medical Services, Board of Public Education, City of Philadelphia; Dr. Joseph S. Kenney, Acting Chief, Parochial Medical Division, Board of Public Education, City of Phila-

delphia; Mr. Emil A. Tiboni, Chief, Community Hygiene Section, Division of Environmental Health, Philadelphia Department of Public Health; Dr. C. J. Gentzkow, Director, Division of Laboratories, Commonwealth of Pennsylvania Department of Health, City of Philadelphia; Mr. James Copeland, Chief, Public Health Laboratory, City of Philadelphia; Dr. Richard Connell, Statistics Section, Communicable Disease Center; and Dr. Jose de Celis, Committee on Puerto Rican Affairs of Philadelphia.

REFERENCES

1. Acosta-Matienzo, J., 1946. Incidence of intestinal protozoa among patients of the University Hospital at San Juan, Puerto Rico. *Puerto Rico J. Pub. Health & Trop. Med.*, **21**: 369–371.
2. American Public Health Association Committee on the Hygiene of Housing, Report on an appraisal method for measuring the quality of housing. A yardstick for health officers, housing officials and planners, 1945.
3. Brooke, M. M., and Goldman, M., 1949. Polyvinyl alcohol-fixative as a preservative and adhesive for protozoa in dysenteric stools and other liquid materials. *J. Lab. Clin. Med.*, **34**: 1554–1560.
4. Brooke, M. M., Melvin, D. M., Sappenfield, R., Payne, F., Carter, F. R. N., Offutt, A. C., and Frye, W. W., 1955. Studies of a water-borne outbreak of amebiasis, South Bend, Indiana III. Investigation of family contacts. *Am. J. Hyg.* **62**: 214–226.
5. Dept. of Public Health of Philadelphia, report on Haddington Leadership Area, Housing & Neighborhood Conditions, Opinions and Attitudes of the Residents, 1955.
6. Dept. of Public Health of Philadelphia, report on Hawthorne Leadership Area, Housing & Neighborhood Conditions, Opinions and Attitudes of the Residents, 1957.
7. Faust, E. C., Hoffman, W. A., Jones, C. A. and Janer, J. L., 1934. Studies in *S. mansoni* in Puerto Rico. II. The epidemiology and geographical distribution of *S. mansoni* in Puerto Rico. A survey of intestinal parasites in endemic schistosomiasis areas in Puerto Rico. *Puerto Rico J. Pub. Health & Trop. Med.* **9**: 447–471.
8. Maldonado, J. F., Acosta-Matienzo, J. and Velez, H. F., 1954. Comparative value of faecal examination procedures in the diagnosis of helminth infections. *Exper. Parasitol.* **3**: 403–416.
9. Myers, E. N., Negron, R. and Pearlstein, H., 1959. Intestinal parasitoses in Puerto Rican preschool children at Philadelphia, Pennsylvania, in 1958. *Am. J. Med. Sci.*, **237**: 59–66.
10. Poindexter, H. A., 1933. The Puerto Rican strain of *E. histolytica*. *Puerto Rico J. Pub. Health & Trop. Med.*, **9**: 31–33.
11. Ritchie, L. S., 1948. An ether sedimentation technique for routine stool examinations. *Bull. U. S. Army Med. Dept.*, **8**: 326.
12. Weller, T. H. and Dammin, G. J., 1945. The incidence and distribution of *Schistosoma mansoni* and other helminths in Puerto Rico. *Puerto Rico J. Pub. Health & Trop. Med.*, **21**: 125–147.
13. Young, V. M. and Felsenfeld, O., 1948. The occurrence of intestinal protozoa in adults in San Juan, Puerto Rico. *J. Parasitol.*, **34**: 229–230.

RELATION OF WATER VELOCITY TO *SCHISTOSOMA MANSONI* INFECTION IN MICE

WILLIAM B. ROWAN* AND ANDREW L. GRAM†

Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, San Juan, Puerto Rico

Occasionally, while discussing problems of schistosomiasis transmission in Puerto Rico, the writers have been asked whether a person could swim or bathe in comparative safety in rapidly moving water in rivers and streams where, because of the high water velocity, cercariae might find little opportunity for reaching or penetrating the surface of the skin. Apparently no previous studies of this possibility have been made. Such information might be useful to persons who, through necessity, must use cercaria-infested waters for bathing, clothes washing, or other purposes. Consequently, a study was undertaken to test the effect of water velocity on the establishment of *Schistosoma mansoni* infection in mice. This paper describes the results of the laboratory and field experiments done.

METHODS AND MATERIALS

Of a total of 90 mice used in these experiments, 60 were exposed to cercariae in an especially constructed flow chamber apparatus in the laboratory and 30 were exposed to the water of a naturally infected stream. The flow chamber apparatus consisted of a level, galvanized metal channel 2.54 cm wide, 12.7 cm deep and 3.5 meters long, and a 38-liter plastic container with siphon, mounted on a screw-type lift jack. The channel was supplied with tap water from a nearby faucet. The volume of water entering the channel and hence the velocity of flow in the channel were varied by adjusting the faucet. A movable strip of metal mounted in the channel near the outlet end provided a simple means of maintaining a constant water level in the channel during an experiment. The plastic container held the suspension of cercariae used in each experiment, and this suspension was allowed to siphon continuously into the water channel during the experiment. By adjusting the lift jack beneath the container the siphon head between container and channel was kept constant.

In each experiment involving the flow channel, 5 mice were used. During exposure each mouse was restrained, using a device similar to the one designed by Stirewalt and Bronson,[4] and the tail of the mouse, which protruded, was taped to a wooden applicator stick. The 5 mice were mounted at 5-meter intervals at the surface of the water in the channel so that their tails extended down into the water at right angles to the flow. The exposure time for the laboratory experiments was 30 minutes, and after exposure the 5 mice were maintained together in a single cage to allow time for development of the worms. The 5 mice comprising a group were either all males or all females varying in age from 6 to 10 weeks. All came from laboratory colonies of unknown lineage.

The laboratory experiments were conducted on 3 different days, 4 each day. The procedure on each day was as follows:

1. A 20-liter suspension of freshly shed cercariae in aerated tap water was prepared in the plastic container, the concentration of cercariae being adjusted to approximately 9000 per liter. The plastic container was mounted on the lift jack and the siphon tube (which delivered 0.2 liter per minute) was suspended over the edge of the container so that its outlet end extended into the channel near the latter's inlet end.

2. The channel was filled to a depth of 10.4 cm (experiments 1 to 8) or 6.4 cm (experiments 9 to 12). Then, by gradual adjustments of the metal strip mounted in the channel so as to hold the depth constant, and by slight adjustments of the faucet, the volume of flow (as determined by timing the outflow) was stabilized at approximately 316 ml per second. This was the greatest constant volume of flow which could be achieved with the city water pressure available.

3. The velocity of flow of the water in the channel was measured directly at this time and

* Present address: Department of Biology, Middlebury College, Middlebury, Vermont.
† Present address: Engineering Science, Inc., 490 East Walnut Street, Pasadena, California.

at the end of each experiment by observing the rate of passage of a few drops of ink from one part of the channel to another.

4. Once the mice were positioned, the cercarial suspension in the plastic container was stirred and the connecting siphon was started. Stirring of the cercarial suspension continued throughout the exposure period. The outlet of the siphon extended under the surface of the water in the channel and the siphon head was maintained by means of the jack at approximately 15 cm. During the 30-minute exposure period, two 3.8-liter water samples were collected at the outlet of the channel for cercarial density determinations.

5. After 30 minutes, the siphon was stopped, stirring of the cercaria suspension ceased, and the 5 mice were removed from their positions and returned to the animal room.

6. The velocity and volume of flow were again measured and recorded.

7. The procedure was the same for the remaining 3 experiments. With each experiment, the volume of flow in the channel and, hence the velocity of flow, were reduced by one-fourth. The cercaria suspension remaining after each experiment was diluted, using aerated tap water from the same source, so that the cercarial density at each new flow velocity remained unchanged. For experiments 2, 3, and 4, the remaining suspension was diluted by one-third, one-half, and one part of water, respectively.

Cercarial density determinations of the 3.8-liter samples were made using a modification of the sampling technique described by Rowan.[2, 3] Instead of pressure filtration, a Büchner funnel (perforated plate 111 mm in diameter) and filtering flask (4-liter capacity) were used. The water sample collected from the channel was poured immediately over filter paper (S & S 404, 110 mm in diameter) in the Büchner funnel, and gentle suction was created by a hand suction bulb (with valve) attached to the filter flask. This forced the water through the paper, leaving the cercariae trapped on the paper. After the entire sample had been filtered, the damp paper was placed on top of 2.0 ml of 0.5 per cent ninhydrin stain in a shallow pan and heated to dryness over a Bunsen burner. The cercariae on the dry paper were observed by placing the paper between glass plates, saturating it with xylol containing excess sodium bicarbonate, and examining the paper, using a dissecting microscope and reflected light. As is usual with this technique, some of the cercariae appear well oriented on the paper and some are broken or lie partly buried in the fibers. Based on 10 trials using the Büchner funnel technique and a sample volume of 3.8 liters, it was learned that essentially 100 per cent of the cercariae in a sample are recovered on the paper and 73±4 per cent of the total number in the sample are sufficiently well oriented on the paper to be easily recognized as *S. mansoni*.

The field experiments were conducted in a small stream which flows through the community of Faria near Bayamon, Puerto Rico. The section of stream studied was approximately 10 meters long. Most of the infected snails were located just upstream from this section in slow moving water. Downstream towards the study section, the stream bed gradually narrowed and the water velocity increased. By using red dye, the average velocity for 1.5 meters was determined at three stations in the 10-meter section. Ten mice were exposed in midstream at each of these 3 stations on April 15, 1958 for 2 hours from 11 a.m. to 1 p.m. The exposure was accomplished by carefully suspending cages in the water in such a way that the mice were forced to remain partly immersed in the water at all times. The cercarial density of the water was measured 3 times at each station during the 2-hour period using the pressure filtration apparatus with "direct sampling" of 19 liters (5 gallons) of water.[2, 3] Since few cercariae were found to be present in the water on this day, the same mice were exposed at the same positions for 2 additional hours the following day (11 a.m. to 1 p.m.) and 3 more cercarial density determinations were made. Therefore, the total exposure time for these mice was 4 hours.

The liver and intestine perfusion technique[1] was used to recover the adult worms from infected mice. The worms collected were fixed in cold AFA fixative and counted. The mice used in experiments 1 to 8 and 13 to 15 were examined 8 weeks after exposure. Some of the mice used in experiments 9 to 12 appeared too sick at 6 weeks to survive much longer and the entire series was sacrificed 45 days after exposure.

RESULTS

The results of the 15 experiments are summarized in Table 1. The second column of the table gives the volume of flow of water through

TABLE 1

Relation of water velocity to Schistosoma mansoni *infection in mice*

1		2	3	4	5	6	7	8	9	10	11
Experiment		Volume of flow*	Velocity of flow	Area of mouse exposed	Exposure	Water passing exposed mouse's skin during exposure period (3 × 4 × 5)	Average number of cercariae per liter of water	Estimated number cercariae approaching the exposed mouse's skin during exposure period $\left(\frac{6 \times 7}{1000}\right)$	Number and sex of the mice	Average number of adult worms per mouse	Percentage recovery of cercariae as adult worms $\left(\frac{10}{8} \times 100\right)$
No.	Date										
		ml/sec.	*cm/sec.*	*sq cm*	*sec.*	*ml*					
1	4-10-58	316	10.9	1.4	1,800	27,400	75.8	2,080	5F	13.2	0.64
2	4-10-58	237	8.2	1.4	1,800	20,500	79.3	1,630	5F	12.8	0.79
3	4-10-58	158	5.4	1.4	1,800	13,700	77.1	1,060	5F	3.6	0.34
4	4-10-58	79	2.7	1.4	1,800	6,900	75.0	510	5F	2.4	0.47
5	4-11-58	316	10.9	1.4	1,800	27,400	72.4	1,980	5F	11.2	0.57
6	4-11-58	237	8.2	1.4	1,800	20,500	72.4	1,490	5M	12.0	0.81
7	4-11-58	158	5.4	1.4	1,800	13,700	74.2	1,020	5F	4.4	0.43
8	4-11-58	79	2.7	1.4	1,800	6,900	69.0	470	4M	1.0	0.21
9	4-28-58	316	19.6	1.4	1,800	49,300	79.5	3,920	4F	70.0	1.79
10	4-28-58	237	14.7	1.4	1,800	37,000	95.4	3,530	5M	49.4	1.40
11	4-28-58	158	9.8	1.4	1,800	24,700	88.8	2,190	5M	16.2	0.74
12	4-28-58	79	4.9	1.4	1,800	12,300	81.9	1,010	3F	1.7	0.17
13	4-15-58	—	50.0†	6.45	14,400	4,644,000	0.705	3,270	5M, 5F	44.5	1.36
14	4-15-58	—	10.6†	6.45	14,400	983,000	0.354	350	5M, 5F	1.2	0.34
15	4-15-58	—	4.2†	6.45	14,400	394,000	0.428	170	10M	0.4	0.24

* Volume of flow, cross sectional area of channel: experiments 1 to 8, 29.03 sq cm; experiments 9 to 12, 16.13 sq cm.
† Measured velocity.

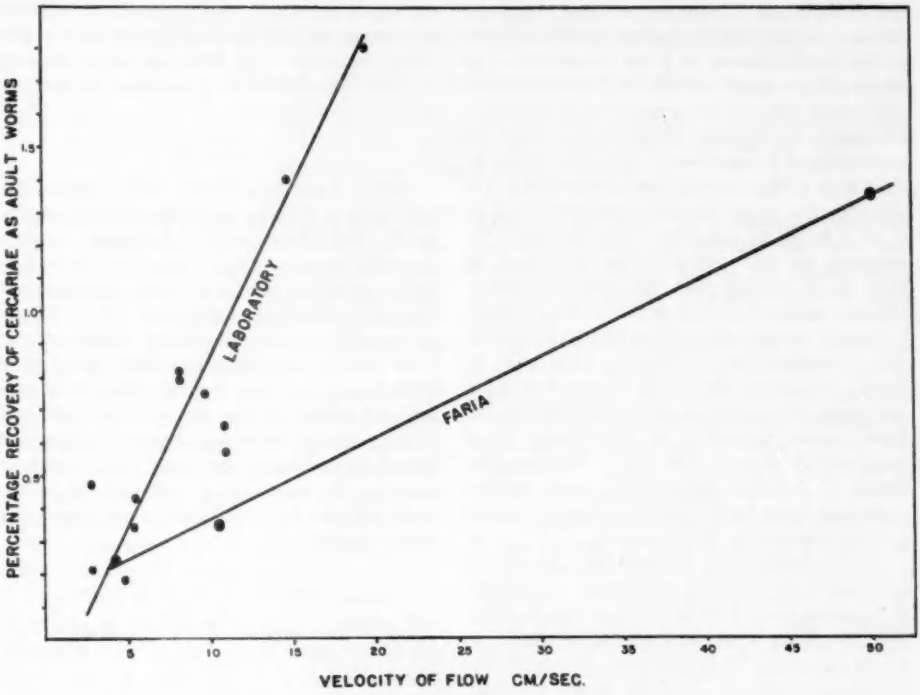


FIG. 1. Percentage recovery of cercariae as adult worms

the laboratory channel as measured at the outlet. The calculated velocity of flow for experiments 1 to 12 (volume of flow divided by cross sectional area of channel) and the measured velocity for experiments 13 to 15 are given in column 3. The measured velocities for experiments 1 to 12 compared favorably with the calculated velocities, but, because of the uniformity of the channel and the accuracy with which the volume at the outlet could be measured, the calculated velocities are considered the more accurate. The units in column 4 supposedly represent the area of mouse skin exposed on the upstream side. The product of velocity times exposed skin area times seconds of exposure provides a rough measure of the volume of water actually passing the exposed skin of each mouse during the experiment (column 6) and this volume multiplied by the cercarial density gives an estimate of the number of cercariae which actually might have approached the exposed mouse skin during the experiment (column 8). Finally, the average number of adult worms recovered, divided by the estimated number of cercariae approaching multiplied by 100 (column 11) is taken as a rough estimate of the percentage recovery of cercariae as adult worms. The relationship between velocity of flow of the water and percentage recovery is shown in Figure 1.

It is evident from Table 1 that, within the limits of these experiments, the worm burden increases with increasing velocity. The data also show a significant increase with velocity in percentage recovery of cercariae as adult worms.

DISCUSSION

According to these data, if the cercarial density remains constant, mice acquire heavier worm infections in fast water than in slow water. This result is not unreasonable since, per unit time, more cercariae pass the mouse in fast water than in slow water. The velocities used in experiments 1 to 12 were impressive when viewed in the laboratory, but actually they fall at the lower

end of the range of velocities common to natural streams on the island. Shallow rapids occurred at the lower station at Faria (experiment 13) where the measured velocity of flow was 50 cm per second. One factor common to the 3 series of laboratory experiments should be mentioned. In each series of 4 experiments, it was necessary to start with a high velocity and proceed to a low velocity. The 4 experiments required a total of 3 or 4 hours to complete, including time for preparing the mice and adjusting the volume of flow. It is possible that the cercariae, under constant agitation caused by the stirring, became "fatigued" in some way during this time interval with a resulting loss of ability to infect mice. If fatigue did play a role in the 4 experiments for any given day, one would expect proportionately lower worm recoveries in experiments done progressively later in the day. However, the results of the field experiments, where fatigue could not have been a factor, conform to the pattern observed in the laboratory.

An interesting result observed from the data is the relationship between velocity and percentage of cercariae recovered as adult worms in mice (Fig. 1). Many theoretical objections might be raised regarding the method used in calculating percentage recovery. Perhaps more practical methods will develop as additional thought and research are given to problems of this type. Two explanations might be advanced to explain the trend observed. It is possible that cercariae are stimulated to a greater extent to seek a point of attachment in fast water as compared with slow water. It is also possible that the thinner boundary layer of still water near the tail resulting from increased velocity is more easily penetrated by cercariae in the vicinity of the tail. Once again, however, a technical objection can be advanced. In heavily infected mice the veins of the liver and intestine are distended. It is possible that the perfusion technique for recovering worms from these veins becomes more efficient the heavier the infection. If so, this shift in efficiency inherent in the technique might account for the disproportionate percentage of recovery at the higher velocities.

SUMMARY

Twelve laboratory and 3 field experiments were done to observe the effect of water velocity on the establishment of *Schistosoma mansoni* infections in mice. Ninety mice were exposed to water containing from 0.35 to 95.4 cercariae per liter with velocities ranging from 2.7 to 50 cm per second. Infections developed in the mice at every velocity studied. From these preliminary experiments it would appear that, when the cercarial density of the water is held constant, mice exposed to fast water acquired more worms than those exposed to slow water. The percentage recovery of approaching cercariae as adult worms in mice increased with an increase in the water velocity.

ACKNOWLEDGMENTS

The writers wish to thank Mr. Marcos Diaz and Mr. Tomas Ocasio (Department of Health, Commonwealth of Puerto Rico) for their technical assistance during this study.

REFERENCES

1. Radke, M. G., Berrios-Duran, L. A., and Moran, K., 1957. A perfusion procedure (Perf-O-Suction) for recovery of schistosome worms. *J. Parasitol.* **43**(5, Section 2): 26.
2. Rowan, W. B., 1957. A simple device for determining population density of *Schistosoma mansoni* cercariae in infected waters. *J. Parasitol.* **43**: 696–697.
3. Rowan, W. B., 1958. Daily periodicity of *Schistosoma mansoni* cercariae in Puerto Rican waters. *Am. J. Trop. Med. & Hyg.* **7**: 374–381.
4. Stirewalt, M. A., and Bronson, J. F., 1955. Description of a plastic mouse restraining case. *J. Parasitol.* **41**: 328.

MALARIA SURVEILLANCE IN THE UNITED STATES, 1958

JACOB A. BRODY, AND FREDERICK L. DUNN

Epidemic Intelligence Service Office, Surveillance Section, Epidemiolgy Branch, Communicable Disease Center, Bureau of State Services, Public Health Service, United States Department of Health, Education, and Welfare, Atlanta, Georgia

In 1958, for the first time since malaria morbidity reporting was established, fewer than 100 cases were reported in the United States. Seven of these cases were of apparent indigenous origin, 4 as a result of blood transfusions. The cases of foreign origin were divided equally between the military and civilian populations.

The United States is well advanced in the third stage of malaria eradication.[10] Indigenous malaria has almost disappeared but the need for constant surveillance remains to prevent the re-establishment of endemic foci in the face of an abundant anopheline mosquito population.

The Communicable Disease Center (CDC), through a series of publications, has documented the decline of malaria in the United States.[1-6, 8, 9] In 1935, over 150,000 cases of malaria were reported, whereas for 1945 the figure was 60,000. In 1955 there were 522 reported cases and by 1957 the number of cases had fallen to 132.[7]

A brief accounting of the 1958 malaria experience will be presented below, with particular attention being given to the naturally acquired and transfusion-acquired indigenous cases. The data in this report were secured through the cooperation of the State Health Departments, the National Office of Vital Statistics (NOVS), and the National Repository for Malaria Slides, CDC, Chamblee, Georgia. Without the full support of these agencies, this analysis would not be possible.

APPRAISAL OF CASES; 1958

The Surveillance Section of CDC received reports of 94 cases of malaria during 1958. The provisional number reported by the NOVS for the year was 81, the discrepancy resulting from the fact that several states do not report cases clearly of foreign origin to NOVS.

Of the 94 cases reported to CDC, 61 were confirmed by adequate clinical, laboratory, and epidemiologic evidence. Eleven cases were presumptive, with clinical and epidemiologic evidence suggestive of malaria, but laboratory evaluation in doubt. For 17 cases the diagnosis was not confirmed due to insufficient data, and 5 cases were judged not to be malaria when evidence of another diagnosis came to light (Table 1).

The cases were reported from 23 states and the Territory of Hawaii. As in 1957, California had the highest state incidence with 25 cases. Other states with relatively large numbers of cases included New York (11 cases, 8 from New York City), Indiana and North Carolina (6 cases), and Illinois (5 cases).

The geographic distribution in the continental United States of confirmed and presumptive cases is shown in Figure 1. It is clear that cases were reported throughout the nation in a distribution totally different from that observed during the years when indigenous malaria was a major problem in the United States. The current distribution is largely a reflection of the dispersion of people who have returned, infected, from malarious areas of the world.

The quarterly distribution of reported malaria for 1958 by date of onset was 10 cases in the first quarter, 22 in the second, 19 in the third, and 13 in the last. As would be expected, no very sharp peak occurred during the anopheline breeding season in the United States since the overwhelming majority of cases were acquired abroad.

Laboratory diagnosis by species was available for 60 of the confirmed and presumptive cases. *Plasmodium vivax* was described in 53 instances, *P. malariae* was diagnosed 6 times and *P. falciparum* once.

ORIGIN OF CASES

Of the 72 confirmed and presumptive cases, 65 were infected in foreign areas and 7 were indigenous (Table 1). A total of 33 cases occurred in military personnel and in veterans who acquired their initial infection while in the Armed Forces. All of these cases were of foreign origin, most of the infections having been acquired in

eastern Asia. Of 28 cases for which country of origin is known, 9 were contracted in Korea. In 9 instances cases were believed to have originated in the Philippine Islands although in 3 of these cases the disease may have been acquired in Okinawa (Table 2).

TABLE 1

Malaria surveillance in the United States, 1957–1958

	1957	1958
Total reports to NOVS	132	81
Appraised by CDC	138	94
Confirmed	102	61
Presumptive	6	11
Not confirmed	25	17
Not malaria	5	5
Total confirmed and presumptive	108	72
Indigenous cases	11	7
Foreign acquired cases	97	65
Military	56	33
Civilian	41	32

Of the 32 civilian cases, 7 originated in Mexico. During the previous year, 15 illnesses were believed to have been contracted in Mexico. The decline in recent years in cases of Mexican origin appears to be a reflection of progress in the malaria eradication program in Mexico. The remaining 25 civilian cases originated in 16 different countries scattered throughout the world: 6 in Asia, 5 in Africa, and 10 in Latin America exclusive of Mexico (Table 2).

INDIGENOUS CASES

Seven cases of confirmed and presumptive malaria were apparently acquired in the United States during 1958. Four of these cases were associated with blood transfusions, and in three instances the infection was naturally acquired. The indigenous cases were not in any way associated with each other; each was an independent occurrence, and investigation has failed to reveal any actual foci of infection.

A. Natural Infections

1. A 10-year-old female with vivax malaria was reported by the Sacramento County Health

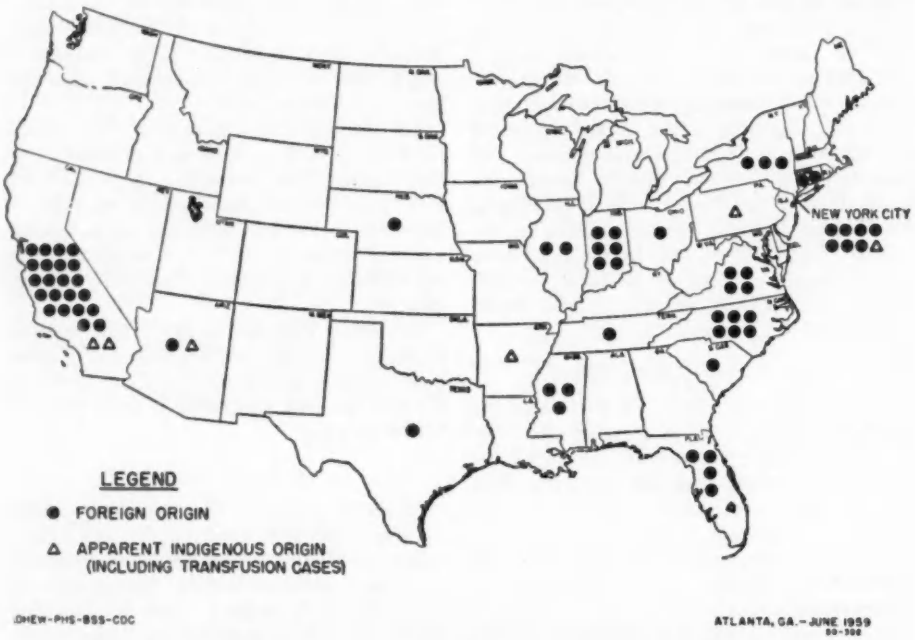


FIG. 1. Distribution of confirmed and presumptive cases appraised by CDC (71 cases)

TABLE 2

*Malaria surveillance in the United States, 1958: Foreign sources of cases**†*

Country	Military	Civilian
Korea	9	1
Philippine Islands	9	
Okinawa	4	
Guam	1	
China	1	
Thailand	1	
South Pacific		1
India		1
Pakistan		3
Morocco	2	1
Kenya		1
Africa		2
Algeria		1
Mexico		7
Honduras		3
Panama	2	1
El Salvador	1	1
Puerto Rico		1
Brazil		1
Colombia		1
Venezuela		1
South America		1

* Data supplied by States to the Communicable Disease Center.

† Where origin may have occurred in one of two countries both countries are listed.

Department in California, and the diagnosis was confirmed by the laboratory of the California State Department of Public Health. The girl became ill on December 10, 1958, with characteristic chills and fever which responded to antimalarial treatment. The exact location of origin of the infection cannot be determined, but three possibilities exist. The child vacationed during the last 2 weeks of July at a camping site in Sierra County, along the Yuba River. She also visited her grandmother in Esparto, Yolo County, where there are many Mexican migrant laborers, and anopheline mosquitoes are abundant. Finally, the infection may have been acquired in the vicinity of her home in Sacramento. Unless other cases should appear in one of these areas, the exact origin of this infection will remain in doubt.

2. A 54-year-old male with a diagnosis of vivax malaria was reported by the Arizona State Department of Health, from Douglas County, Arizona. The diagnosis was made by a local physician on the basis of a blood smear. The slide was not available for review by the State Department of Health or the National Repository for Malaria Slides. The patient became ill about October 1, with periodic chills and fever at 5-day intervals. He was treated for an ear infection. The chills and fever persisted and the patient was hospitalized from October 31 to November 18. He was treated with chloroquine from November 14 to November 18.

The family of the patient had previously complained to the local health authorities of numerous pest mosquitoes. The patient gave no history of travel or contact with veterans or persons with malaria. No other cases in the area were reported.

3. A 39-year-old male with malaria was reported by the Cambria, Pennsylvania Health Department. The diagnosis was made from smears reviewed by several pathologists and the Chief of the Epidemiology Section of the Pennsylvania Department of Health, but the species was not determined. On July 27, the patient had driven to Gainesville, Georgia, from his home in Pennsylvania. On August 9, the day he returned to Cambria, he became ill. On August 10, he was admitted to the hospital with severe headache and fever, and a diagnosis of influenza. Later that day the diagnosis of malaria was made from a smear and the patient was treated with chloroquine. His temperature remained between 99 and 100°F until August 14, when it rose to 101.6°F. Subsequently it fell to normal. A blood smear obtained on August 15 was read as negative.

The place of acquisition of this infection is not clear. There is no history of travel abroad in recent years. Since malaria of any type is unlikely to become symptomatic before 12 days following infection, it seems that the disease would have had to be contracted in Pennsylvania or en route to Gainesville. In addition, according to officials of the Georgia Department of Public Health, who investigated the Gainesville area thoroughly, the altitude of Gainesville is not conducive to anopheline breeding and no cases of malaria have been recorded from that area for at least 25 years.

B. Cases Associated with Blood Transfusions

1. The New York City Department of Health has reported a case of transfusion malaria in a 56-year-old male who received a blood transfusion

on January 17, 1958, following a fracture of the femur. Diagnosis of *P. malariae* was confirmed by the Tropical Disease Laboratory of the City of New York, which reviewed the smear.

The patient had a spiking fever from February 17 to March 23, when he was hospitalized. From March 23 to April 4 his temperature was normal and 3 blood smears were read as negative. He was discharged from the hospital and readmitted on April 29. On May 3, a blood smear was positive and a regimen of chloroquine followed by primaquine was instituted. The temperature rose to 105°F on May 5, and on May 8 to 101°F. Thereafter his temperature remained normal.

In following up the case, a 28-year-old male who had donated blood used by the patient was found to be infected with *P. malariae* by the Tropical Disease Laboratory. This man had no history of clinical malaria and had resided in New York since 1952 when he arrived from Puerto Rico. The blood, which was donated approximately 24 hours before its use, had been kept under regrigeration.

2. A case of transfusion malaria in a 50-year-old female was reported by the Los Angeles County Health Department. The case was confirmed by the California State Department of Public Health as *P. malariae*. The patient, an alcoholic with periodic bleeding varices, had received some 75 transfusions in recent months, 35 of which were administered during a hospitalization immediately before the time of diagnosis of malaria. With this prodigious number of transfusions, no attempt to identify the infected donor was undertaken.

3. A 62-year-old male was reported from Poinsett County, Arkansas, by the State Board of Health as having developed transfusion malaria. The diagnosis of *P. vivax* malaria was confirmed by the University of Arkansas Medical Center. The patient received transfusions associated with a partial gastrectomy and esophogectomy performed on October 2, 1958, for gastric carcinoma. Six pints of blood were administered, one on September 18, one on September 20, and four on October 2. He developed symptoms of malaria on October 10, 1958. The patient gave a history of having malaria at age 7 or 8. Slides from all donors were examined by the Arkansas State Board of Health and found to be negative.

4. One case of transfusion malaria from the Territory of Hawaii was reported. Details are not available at the time of this writing.

SUMMARY AND CONCLUSIONS

Ninety-four cases of malaria were reported to the Communicable Disease Center during 1958 from 23 states and Hawaii. Confirmed and presumptive cases numbered 72. Of these, 65 were of foreign origin. Thirty-three of these cases were in military personnel, the majority contracting their illness in eastern Asia (Korea 9, Philippines 9). A total of 32 civilian cases of foreign origin came from 17 different nations. Mexico with 7 cases still leads the list although the numbers of cases acquired in Mexico has been dropping rapidly in recent years. The malaria eradication campaign in Mexico is considered to be a major factor in this decline.

Only three naturally acquired indigenous cases were reported—one from California, one from Arizona, and one from Pennsylvania. The actual places of origin of these cases could not be determined and no associated cases or endemic foci were found.

Four cases apparently resulting from blood transfusions were reported. A blood donor for one of these cases was identified as having a silent *P. malariae* infection, implying that, in 1958, the risk of acquiring malaria in the United States was greater from blood transfusions than from natural causes. The occurrence of these transfusion-acquired cases suggests the possibility that there may be numerous silent malaria infections in this country. These silent parasitemias do not, however, appear to be an important source of plasmodia capable of introducing the infection into the mosquito population and creating endemic foci.

As long as malaria prevails in other parts of the world, the threat of malaria outbreaks in the United States remains. Anopheline mosquitoes are abundant and their opportunities for survival increase with the increasing amount of land under irrigation. Only through careful surveillance of malaria and investigation of each reported case with the promptness and vigor customary in the investigation of cases of smallpox or plague can we preserve the near freedom from malaria we are currently enjoying.

REFERENCES

1. Andrews, J. M., 1948. What's happening to malaria in the U. S. A.? *Am. J. Pub. Health* **38**: 931–942.

2. ANDREWS, J. M., AND GILBERTSON, W. E., 1950. Final phases of malaria eradication in the United States. *J. Nat. Malaria Soc.* **9:** 5–9.
3. ANDREWS, J. M., QUINBY, G. E., AND LANGMUIR, A. D., 1950. Malaria eradication in the United States. *Am. J. Pub. Health* **40:** 1405–1411.
4. ANDREWS, J. M., 1951. Nationwide malaria eradication projects in the Americas. I. The eradication program in the U. S. A. *J. Nat. Malaria Soc.*, **10:** 99–123.
5. DUNN, F. L., AND BRODY, J. A., 1959. Malaria surveillance in the United States, 1956–1957. *Am. J. Trop. Med. & Hyg.* **8:** 447–455.
6. FRITZ, R. F., AND ANDREWS, J. M., 1953. Imported and indigenous malaria in the United States, 1952. *Am. J. Trop. Med. & Hyg.* **2:** 445–456.
7. NATIONAL OFFICE OF VITAL STATISTICS, Public Health Service, U. S. Dept. Health, Education, and Welfare, Washington, D. C., 1959. Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 6, No. 53, October 29.
8. QUINBY, G. E., 1950. Epidemiological appraisal of reported malaria in the United States from 1947 through 1949. *J. Nat. Malaria Soc.* **9:** 397–400.
9. WELCH, S. F., AND QUINBY, G. E., 1952. Epidemiologic appraisal of malaria in the United States during 1951. *Am. J. Trop. Med. & Hyg.* **1:** 736–742.
10. WORLD HEALTH ORGANIZATION CHRONICLE XIII. Malaria eradication. No. 2, 71–73, 1959.

INTRADERMAL TESTS WITH *DIROFILARIA IMMITIS* EXTRACT IN EOSINOPHILIC LUNG (TROPICAL EOSINOPHILIA)

T. J. DANARAJ AND JOHN F. SCHACHER*

Departments of Medicine and Parasitology, University of Malaya, Singapore

Although patients suffering from eosinophilic lung (tropical eosinophilia) neither give a history of filariasis nor have the accepted signs of this infection, and examinations of blood do not reveal microfilariae, the presence of complement-fixing antibodies in their sera has been demonstrated by using as antigen an alcoholic extract of dried powder of *Dirofilaria immitis*.[1, 2] In view of this finding and the satisfactory response of patients to treatment with diethylcarbamazine, the etiology of a filarial infection with a species whose normal host may not be man was postulated. In the present study, intradermal injections of a saline extract of the same antigen were used to ascertain the skin reaction in patients with eosinophilic lung and in control groups. The results of the test indicate a high positive rate in this condition.

MATERIAL AND METHODS

The worm material used in antigen preparation was supplied by Professor Paul C. Beaver, Tulane University School of Medicine, New Orleans, Louisiana. Living *D. immitis* adults were washed in saline and homogenized in distilled water in a Waring Blendor. This material was filtered through gauze, dispensed into ampules, shell-frozen in alcohol-CO_2-ice, then dried and sealed *in vacuo*. Each ampule contained approximately 0.1 gm of dried material, the equivalent of about six worms. This powder was thoroughly ground in a mortar and extracted with sufficient normal saline to make a 1:100 dilution. This was incubated for 2 hours at 37°C with frequent shaking. After 16–20 hrs. at room temperature (26–30°C), the suspension was centrifuged for 30 minutes at 2500 rpm, the supernatant was filtered through Whatman No. 1 filter paper and then passed through a Seitz asbestos filter. This stock extract was kept in a refrigerator and further diluted when used.

To exclude false positive reactions caused by protein from the dog host carried over with the *D. immitis* worms, serum from an uninfected dog was used as control.[3, 4] A 1:100 dilution of dog serum in normal saline was prepared and passed through a Seitz asbestos filter. This stock solution was kept in a refrigerator and further diluted before the test.

Preliminary tests using a 1:8000 dilution of the antigen extract produced severe reactions in some cases of filariasis and gave a large number of false positive reactions in control individuals. The tests were repeated using dilutions of the antigen extract ranging from 1:16,000 to 1:64,000 in order to determine the optimal dilution, as advocated by Bozicevich and Hutter.[3] It was found that at a dilution of 1:48,000, the antigen gave the greatest number of true positive reactions, but no severe reactions, in confirmed cases of filariasis, and the least number of false positive reactions in noninfected and unexposed individuals. Therefore, a 1:48,000 dilution of the antigen extract was used for the test on the study groups.

Intradermal injections of 0.05 ml of a 1:8000 dilution of dog serum and 0.05 ml of a 1:48,000 dilution of antigen extract were made into the skin of the anterior surface of the right and left forearms respectively. The wheals obtained were measured in 2 directions immediately after the injection and the average of the 2 readings was taken as the diameter of the wheal; this was repeated 20 minutes later. A reaction was considered positive if the test wheal had increased in average diameter by 3 mm or more by the end of 20 minutes. Erythema and pseudopodia were ignored. To detect sensitivity to the antigen in individuals also sensitive to dog serum, the criterion was an increase in size of the test wheal to a diameter 3 mm or more greater than the diameter of the control wheal at the end of 20 minutes.

The intradermal test was carried out on the following study groups: (1) 69 persons suffering from cough and hypereosinophilia of 3000 or more cells per cu mm diagnosed as eosinophilic

* Present address: Department of Tropical Medicine and Public Health, Tulane University School of Medicine, New Orleans, Louisiana.

TABLE 1

Results of intradermal tests

Groups	No. of Cases	No. with microfilaraemia	No. with positive FCFT	Intradermal test % positive
(1) Eosinophilic lung.....	69	None	69	100.0
(2) Mild eosinophilia....	32	None	None	59.4
(3) Filariasis...	49	17	12	73.5
(4) Normal Asians....	75	None	None	53.3
(5) Normal Britishers.	66	Not examined	Test not done	4.5

lung; (2) 32 persons with mild eosinophilia of varying degree, the counts ranging from 500 to less than 3000 cells per cu mm. These persons were asymptomatic and the eosinophilia was associated with intestinal helminthic infections; (3) 49 cases of human filariasis; (4) 75 normal Asian controls who had no eosinophilia; (5) 66 normal British controls.

Groups (1) to (4) comprised persons from the indigenous population who had lived for many years in Singapore, an endemic area of bancroftian filariasis.[5] The filarial complement fixation test (FCFT) was performed on all persons belonging to these four groups according to the technique described previously.[2] In addition, blood samples taken at night, once in each case using Knott's concentration technique,[6] were examined for microfilariae. No treatment was given to persons in groups (1) to (3) until these tests had been carried out.

Group (5) consisted of individuals who had arrived directly from the United Kingdom and had been resident in Singapore or Malaya for periods varying from three months to less than two years. Their history and physical examination were not suggestive of either eosinophilic lung or filariasis, but no blood examinations were done.

The test was also carried out on a separate group of 95 cases of eosinophilic lung after treatment with diethylcarbamazine at a dosage of 6 mg per kg body weight 3 times a day for 5 days.

RESULTS

The intradermal test performed on 95 cases of eosinophilic lung, who had been cured with diethylcarbamazine, showed a positive rate of 98.9 per cent. These patients initially had a positive FCFT, but after treatment the titers had gradually diminished and were negative at the time the intradermal test was done. Conversion of the FCFT to negative after treatment occurred within 10 weeks in the majority of cases.

The results obtained in this study show that (1) there is a distinct difference between the positive rate of the normal control group drawn from the indigenous population and the British controls who were relatively recent arrivals to the country, (2) the rate obtained for the mild eosinophilia group who were not considered to be suffering from either eosinophilic lung or filariasis is similar to that obtained for the normal Asian group, 59.4 per cent and 53.3 per cent respectively, (3) although the filariasis group gave a higher positive rate than the two normal groups, it was only with the cases of eosinophilic lung that a 100 per cent positivity was obtained, and (4) the high positive rate persisted for the eosinophilic lung group even though the FCFT had become negative following treatment (Table 1).

DISCUSSION

The results of intradermal tests with extracts of *D. immitis* antigen in human filariasis have been reported by many investigators, but the results obtained are not comparable because the technique of antigen preparation differed, the amount and strength of the solution used in the test varied, and different criteria of positivity were employed.[3, 4, 7-20] Nevertheless, it is apparent from a study of these reports that skin sensitivity to *D. immitis* antigen was present in a large number (given variously as 54 per cent to 91 per cent) of persons suffering from filariasis; in the present study a positive rate of 73.5 per cent was obtained. Positive results found in normal individuals drawn from indigenous populations of endemic areas of filariasis are difficult to evaluate and are considered to represent past or latent filarial infection. Lampe[20] has shown that sensitivity to the test is a common occurrence among people exposed to infection although free from clinical symptoms.

The percentage of persons with skin sensitivity to *D. immitis* antigen increases with the length of exposure in an area of endemic filariasis.[16, 17] The Britishers who were relatively

new arrivals to Singapore gave a low positive rate of 4.5 per cent as compared with the normal Asians. With longer periods of residence, British persons would presumably give a higher rate of positive findings which might approximate that of the indigenous population. The similarity in the positive rates of the Asian controls and mild eosinophilia cases, 53.3 per cent and 59.4 per cent respectively, suggests that positive skin tests may be expected in 50 to 60 per cent of the local population in an endemic area and, therefore, the test cannot be of value in the diagnosis of either filariasis or eosinophilic lung.

Conversion of the FCFT to negative occurred when patients with eosinophilic lung were treated with diethylcarbamazine, but the skin sensitivity persisted in 98.9 per cent of them. In cases of filariasis, a positive skin reaction has been present even as late as two years after the end of medication.[27, 28]

From an etiologic point of view the test is significant in that the 100 per cent positive rate among cases of eosinophilic lung suggests a filarial etiology. However, none of the patients observed in this group or others reported previously [2, 21, 22] gave a past history of filariasis, had any clinical signs of the infection or had microfilariae in their blood. However, owing to the nature of the antigen used, the reaction is group-specific and not species-specific and does not differentiate between infections with the different filarial species, whether of human or animal origin. Taliaferro and Hoffman[7] have suggested that the positive reactions obtained in individuals who have not been exposed to human filariasis might be due to infection with *D. immitis*. Positive skin reactions in persons without collateral evidence of filarial infection have been explained by Augustine and Lherisson[23] as attributable to nonhuman filarial worms producing sensitization in man. Thus the possibility of infection by species of filarial worms normally found in nonhuman hosts must be considered in the etiology of eosinophilic lung. Support for this thesis is given by the FCFT which likewise gave a similar positive rate in 100 per cent of eosinophilic lung patients investigated in this study, whereas the rate was only 24.5 per cent in the group with bancroftian filariasis. A similar low incidence of serologic reactions has been obtained previously[2, 24, 25] in this type of filariasis, the minimal immune response being interpreted as a measure of the success of these parasites in adapting themselves to their normal hosts.[26]

The severity of the tissue response, as shown by the massive eosinophilia and the 100 per cent positive rates obtained with the two tests (as compared with the relatively low rates obtained in human filariasis), indicate an infection in which the host-parasite relationship is abnormal. That the infecting organism probably does not attain sexual maturity is shown by the absence of microfilariae in the circulating blood of patients. It is therefore possible that eosinophilic lung is caused by a species of filaria whose normal host is not man.

SUMMARY

The intradermal test using as antigen a 1 per cent saline extract of *Dirofilaria immitis* powder was performed in Singapore on 69 persons with eosinophilic lung, 32 with mild eosinophilia, 49 with filariasis, 75 normal Asians, and 66 normal Britishers. The test was positive in 100 per cent of the cases of eosinophilic lung, 73.5 per cent of the filariasis group, 59.4 per cent of cases of mild eosinophilia, 53.3 per cent of normal Asians, and 4.5 per cent of the Britishers.

The filarial complement fixation test using a 1 per cent alcoholic extract of the same antigen gave a positive rate of 100 per cent in the eosinophilic lung group, whereas only 24.5 per cent of the filariasis patients gave a positive reaction. Skin sensitivity to *D. immitis* antigen persisted in the cases of eosinophilic lung even when the previously positive serologic reactions had become negative following treatment with diethylcarbamazine. Therefore, the intradermal test cannot be useful in the diagnosis of either filariasis or eosinophilic lung in Singapore.

In view of the skin sensitivity to a filarial antigen demonstrated in patients suffering from eosinophilic lung, the etiologic possibility of an infection by a species of filarial worm found normally in nonhuman hosts is discussed.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Messrs. Ong Soon Lim and Wong Ah Fook for their assistance in carrying out the intradermal tests; to Mr. S. Suppiah for doing the blood counts; and to Mr. Tye Cho-Yook of the Department of Social Medicine and Public Health, University of Malaya for statistical assistance

This investigation was conducted under the sponsorship of the Commission on Parasitic Diseases, United States Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army.

REFERENCES

1. Danaraj, T. J., da Silva, L. S., and Schacher, J. F., 1957. The filarial complement fixation test in eosinophilic lung (tropical eosinophilia). A preliminary report. *Proc. Alumni A. Malaya* **10:** 109–116.
2. Danaraj, T. J., da Silva, L. S., and Schacher, J. F., 1959. The serological diagnosis of eosinophilic lung (tropical eosinophilia) and its etiological implications. *Am. J. Trop. Med. & Hyg.* **8:** 151–159.
3. Bozicevich, J., and Hutter, A. M., 1944. Intradermal and serological tests with *Dirofilaria immitis* antigen in cases of human filariasis. *Am. J. Trop. Med.* **24:** 203–208.
4. Wharton, D. R. A., and Stelma, T., 1946. Comparative cutaneous tests for filariasis with antigens of different dilutions. *J. Infect. Dis.* **78:** 49–59.
5. Danaraj, T. J., Schacher, J. F., and Colless, D. H., 1958. Filariasis in Singapore. *Med. J. Malaya* **12:** 605–612.
6. Knott, J., 1939. A method for making microfilarial surveys on day blood. *Trans. Roy. Soc. Trop. Med. & Hyg.* **33:** 191–196.
7. Taliaferro, W. H., and Hoffman, W. A., 1930. Skin reactions to *Dirofilaria immitis* in persons infected with *Wuchereria bancrofti*. *J. Prevent. Med.* **4:** 261–280.
8. Fairley, N. H., 1931. Serological and intradermal tests in filariasis. A preliminary report. *Trans. Roy. Soc. Trop. Med. & Hyg.* **24:** 635–648.
9. Rodhain, J., and Dubois, A., 1932. A contribution to the study of intradermal reactions in human filariasis. *Trans. Roy. Soc. Trop. Med. & Hyg.* **25:** 377–382.
10. Dickson, J. G., Huntington, R. W., Jr., and Eichold, S., 1943. Filariasis in defence force, Samoan group; preliminary report. *U. S. Naval Med. Bull.* **41:** 1240–1251.
11. Huntington, R. W., Jr., Fogel, R. H., Eichold, A., and Dickson, J. G., 1944. Filariasis among American troops in a South Pacific island group. *Yale J. Biol. & Med.* **16:** 529–537.
12. Michael, P., 1944. Filariasis among navy and marine personnel. Report on laboratory investigations. *U. S. Naval Med. Bull.* **42:** 1059–1074.
13. King, B. G., 1944. Early filariasis diagnosis and clinical findings; report of 268 cases in American troops. *Am. J. Trop. Mep.* **24:** 285–298.
14. Wright, W. H., and Murdock, J. R., 1944. Intradermal reactions following the use of *Dirofilaria immitis* antigen in persons infected with *Onchocerca volvulus*. *Am. J. Trop. Med.* **24:** 199–202.
15. Goodman, A. A., Weinberger, E. M., Lippincott, S. W., Marble, A., and Wright, W. H., 1945. Studies of filariasis in soldiers evacuated from the South Pacific. *Ann. Int. Med.* **23:** 823–836.
16. Huntington, R. W., Jr., 1945. Skin reactions to *Dirofilaria immitis* extract. *U. S. Naval Med. Bull.* **44:** 707–717.
17. Saunders, G. M., Bianco, A. A., and Jordan, W. S., 1946. Intradermal tests with *Dirofilaria immitis* antigen as a diagnostic aid in human filariasis. *U. S. Naval Med. Bull.* **46:** 1242–1253.
18. Zarrow, M., and Rifkin, H., 1946. Observations on the specificity and clinical use of *Dirofilaria immitis* antigen in the diagnosis of human filariasis (*Wuchereria bancrofti*). *Am. J. Med. Sci.* **211:** 97–102.
19. Zeligs, M., 1947. Intradermal tests with *Dirofilaria immitis* extract in human filariasis. *U. S. Naval Med. Bull.* **47:** 824–826.
20. Lampe, P. H. J., 1950. Study on filariasis in Surinam. *Docum. Neerl. et Indones. Morbis Trop.* **2:** 193–208.
21. Danaraj, T. J., 1951. *Eosinophilic Lung. A Study of 150 Cases Seen in Singapore.* Papineau Studios, Singapore, 55 pp.
22. Danaraj, T. J., 1958. The treatment of eosinophilic lung (tropical eosinophilia) with diethylcarbamazine. *Quart. J. Med.* N. S. **27:** 243–263.
23. Augustine, D. L., and Lherrisson, C., 1946. Studies on the specificity of intradermal tests in the diagnosis of filariasis. *Am. J. Hyg.* **43:** 38–40.
24. Ridley, D. S., 1956. The complement fixation test in filariasis. *Trans. Roy. Soc. Trop. Med. & Hyg.* **50:** 255–257.
25. Minning, W., and McFadzean, J. A., 1956. Serological investigations in an area of endemic filariasis due to *Wuchereria bancrofti* and *Acanthocheilonema perstans* in Gambia, West Africa. *Trans. Roy. Soc. Trop. Med. Hyg.* **50:** 246–254.
26. McFadzean, J. A., 1953. Immunity in filariasis of experimental animals. *Am. J. Trop. Med. & Hyg.* **2:** 85–94.
27. Bozicevich, J., 1951. Immunological diagnosis of parasitic diseases. In *Parasitic Infections in Man*, Harry Most, Ed. Columbia University Press, New York, 229 pp.
28. Oliver-Gonzalez, J., 1953. Intradermal reactions to schistosome and filaria antigens in patients before and after treatment of the parasitic infections. *Am. J. Trop. Med. & Hyg.* **2:** 79–84.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN

I. Introduction

W. F. SCHERER and E. L. BUESCHER*

Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

The human epidemics of encephalitis which occur yearly in Japan during August and September are caused by a virus[4, 5, 9] with a complex life cycle involving vertebrates and vector mosquitoes. During summer epidemics and epizootics, virus is readily recoverable in nature from diseased man and animals. Yet the biologic mechanisms responsible for appearance of epidemics and the exact whereabouts of virus during interepidemic periods have been poorly understood. The studies which follow represent an attempt to elucidate the mechanism by which epidemics of Japanese encephalitis (JE) occur. They were initiated in 1952 near Tokyo, Japan by U. S. Army Personnel of the 406th Medical General Laboratory and subsequently were extended into a series of 6 yearly observations of the ecology of JE virus. This introduction presents essential background information and concepts necessary to understand and interpret results of the other articles.

Two principal reasons existed for the decision to study JE in Japan. First, the disease is important to residents of Japan because epidemics of severe human encephalitis occur almost yearly. It was therefore hoped that understanding of the genesis of epidemics might result in control of the disease. Second, an opportunity exists in Japan to study an arthropod-borne virus in *natural pure culture*, free from other coexisting, immunologically related viruses, such as occur in the tropics. This permits extensive use and valid interpretation of simple antibody tests in place of more expensive and time-consuming isolations of virus for detecting asymptomatic infection in animals and man. Two observations provide evidence that the antibodies to JE virus in the habitats to be described near Tokyo resulted from infections by the same and not another immunologically related, but otherwise distinct, Group B arthropod-borne virus. First, the development of antibodies in vertebrates was concomitant with the yearly occurrence of JE as a human disease and with recovery of JE virus from mosquitoes, birds, pigs and man (Articles II, IV, VI and VII). Second, no other immunologically related viruses have ever been recovered near Tokyo by Japanese or American workers. Dengue was found shortly after World War II several hundred miles south of Tokyo in Kyushu and the Kansai region,[3, 7] but it is not a recognized clinical syndrome near Tokyo, nor has dengue virus ever been recovered from natural sources near Tokyo. Thus it seems justified to interpret positive antibody tests of animals and man *resident near Tokyo* during 1952–1956 as indicative of infections by JE virus. Whether antibody in *migratory* adult birds and passively transferred maternal antibody in nestlings are uniformly produced by JE virus is of course another matter, discussed elsewhere.[1] This possibility fortunately is not pertinent to analysis of these studies because birds reported herein were nestlings confined to the study sites near Tokyo where JE virus was the only Group B arthropod-borne virus.

In selecting animals for study, the observations of others were considered along with fundamental biologic concepts. Since *Culex tritaeniorhynchus* had been previously found to be infected by JE virus,[6] it was the principal mosquito studied, although others were also evaluated. In choosing vertebrates for study, attention was directed toward those who, by their short life spans (either natural or induced by slaughter) and high reproductive rates, naturally underwent rapid population turnovers. Such animals, even though infected and immunized annually at high frequencies, offered the greatest potential for provision of susceptible animals to circulate JE virus in blood and infect vector

* Present addresses: Department of Bacteriology and Immunology, University of Minnesota, Minneapolis 14, Minn. (WFS); Dept. of Virus Diseases, Walter Reed Army Institute of Research, Washington 12, D. C. Please send reprint requests for articles of this series to these addresses.

mosquitoes. In Japan the animals that produce large numbers of young each year are birds, wild rodents, pigs and man. Of these, only birds, wild rodents and pigs have a life span less than a few years and thus undergo the required extensive population turnovers. These animals were therefore studied in preference to cows, horses, goats, sheep, dogs and cats whose total and susceptible populations were smaller than those of birds, pigs, rodents and man. Herons and egrets were selected for observation, simply because they possessed JE virus neutralizing antibody,[2] were numerous near Tokyo, were colonial, could be caught as nestlings in large numbers in short periods of time, and were large enough to withstand repeated bleedings of volumes of blood adequate for testing. Their selection is not meant to imply that other birds are not important in JE virus ecology.

STUDIED AREAS

Although these studies were carried out near Tokyo, several facts about Japan as a whole should be mentioned to permit proper extrapolations of findings from one area to another within the country. Japan is a chain of islands with a total land area of 142,000 square miles, slightly smaller than the states of California (158,000 square miles) or Montana (147,000 square miles).[10-12] However, the inhabitable land is only 28.8% of the total; 14.8% being arable, 3.6% pasture, 10.4% covered by buildings, and the remaining 71.2% forest or unexploited land.[8] This means that 90 million people[10] live on 41,000 square miles of land, approximately the area of Ohio (41,222 square miles).[13] Of the total population, 43% are farmers.[8] The inhabitable land hugs the seashore and in certain locations extends inland to form flat plains.

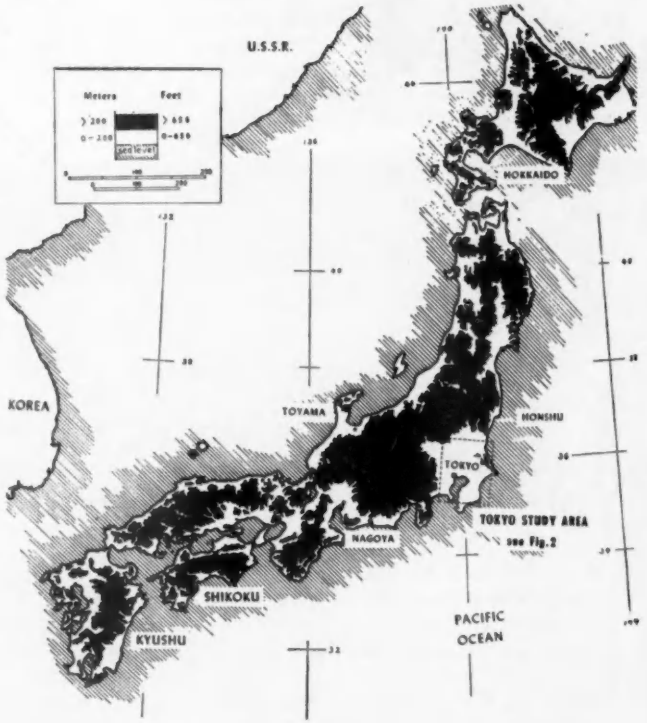


FIG. 1. Habitable areas of the Japanese archipelago

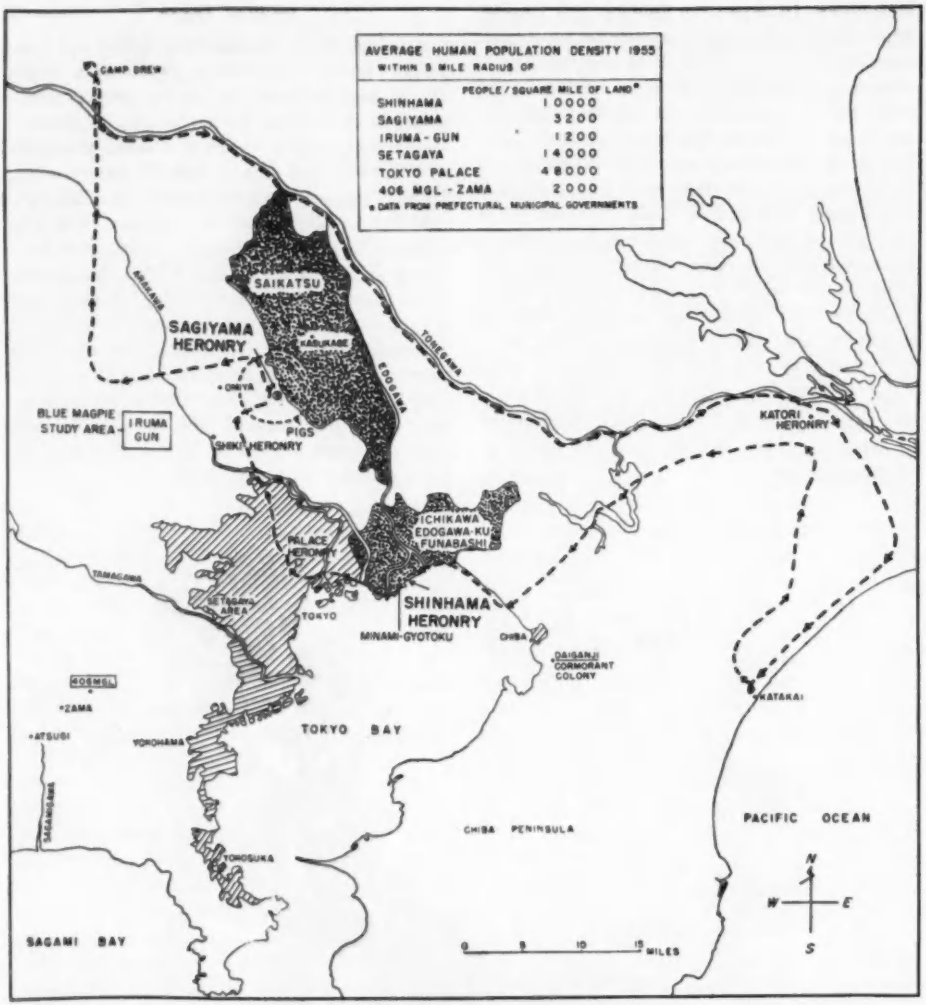


FIG. 2. The Kanto plain, Honshu, Japan, with locations of the principal Japanese encephalitis study sites.

The Kanto plain in which Tokyo exists and where most of these studies were made (Fig. 1), is approximately 3,500 square miles and has a population of at least 12 million people; this represents 9% of the inhabitable land of Japan and 13% of the population. The two major study sites in the Kanto plain were 1) the Shinhama heronry and surrounding region and 2) the Sagiyama heronry and nearby area (Fig. 2). Shinhama, Sagiyama, Minami-gyotoku, the Setagaya district of Tokyo and Zama were sites of mosquito observations. Shinhama, Sagiyama, Irumagun and the Daiganji cormorant colony were areas of bird studies. The heavy dotted line with arrows in Figure 2 shows the route flown over by a helicopter monthly during 1955 and 1956 to evaluate size and behavior of egret populations. Pigs were studied within the circle

around Sagiyama, and in mosquito traps at Sagiyama, Tokyo (Setagaya), Zama and Shinhama. School children were studied at Minamigyotoku and at Kasukabe as samples of humans from the Ichikawa-Edogawa-ku-Funabashi area near Shinhama and from the Saikatsu district near Sagiyama. Wild rodents were trapped south, west and north of metropolitan Tokyo. To emphasize the high human densities in these areas, numbers of people per square mile as of 1955 are listed by regions in Figure 2. For comparison are the followlng 1958 population densities in the U.S.A.: Manhattan Borough of New York City, 90,000 people per square mile of land area; Brooklyn Borough, 35,000[14]; and Washington, D. C., 13,000.[15] Although Sagiyama, Zama and Irumagun were rural areas, they were still more populated than, for example, the states of Rhode Island or New Jersey which had only 748 and 642 people per square mile, respectively, in 1950.[15]

The Shinhama and Sagiyama heronries are shown in Figures 3 and 4, the numbers and letters signifying locations of mosquito traps in 1956. Shinhama is a 17-acre, well-protected duck refuge sponsored by the Imperial Family and located on Tokyo Bay approximately 10 miles east of central Tokyo (Fig. 2). It is less than 10 feet above sea level and is bordered on three sides by rice and lotus paddies (major breeding sites of *C. tritaeniorhynchus*) and on the fourth side by Tokyo Bay. No humans reside in Shinhama although caretakers work there during the day. Relatively few small mammals, notably wild rodents, inhabit the area. Year round, Shinhama abounds with wild waterfowl, herons being the major spring-summer inhabitants and wild ducks being most common in the winter; decoy ducks are raised there throughout the year. Shinhama is bordered by evergreen trees and the fresh water ponds are surrounded by dense bamboo thickets 4 to 10 feet high. During 1952–1957 approximately 2,000 to 4,000 ardeids (chiefly Black-crowned Night Herons, *Nycticorax nycticorax*, Little Egrets, *Egretta garzetta*, and Plumed Egrets, *Egretta intermedia*) inhabited the area during May-September and approximately 200 Little Egrets during the winters. Nests were mostly in bamboo and evergreen trees surrounding the pond, those in bamboo being within easy reach from the ground for capture of nestlings. Birds obtain food (fish, crustacea and amphibians) in nearby paddies and bay, and nestlings are fed

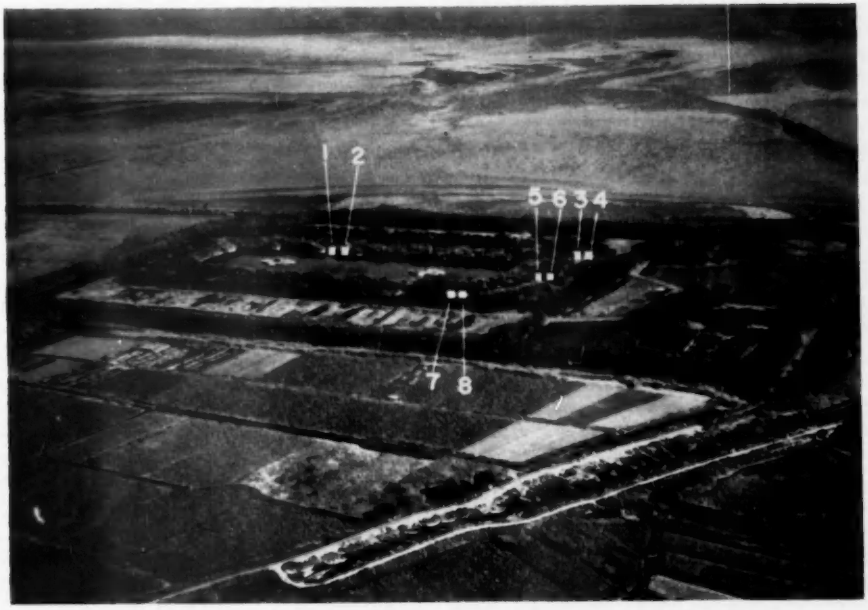


FIG. 3. Shinhama Imperial Refuge. Numbers locate mosquito traps in 1956

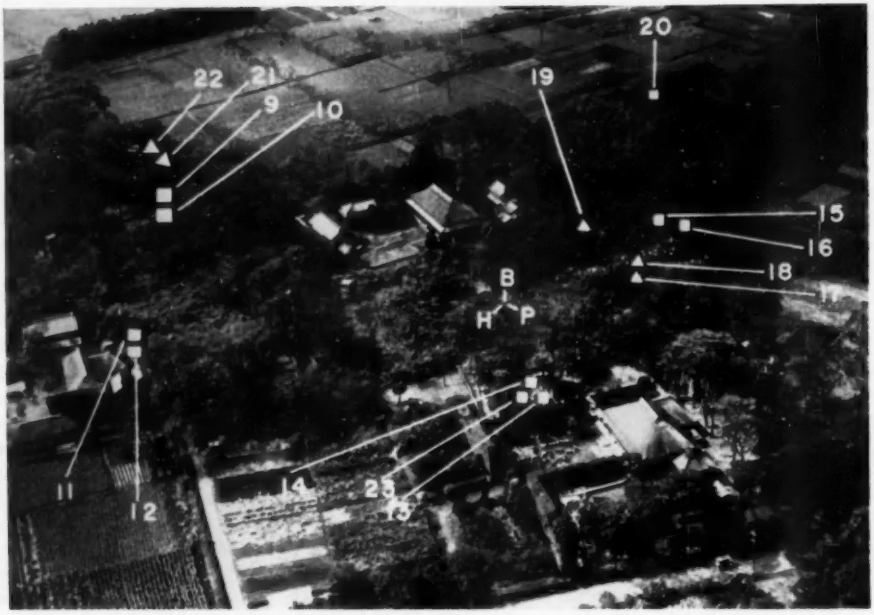


FIG. 4. Sagiyama National Preserve. Numbers and letters locate mosquito traps in 1956

by regurgitation of food from the parents' stomachs or mouths. Although ardeid nests were focally distributed around the pond and during some years, foci of high bird density changed in location during June–August, no great qualitative differences in habitats of nestlings existed. In essence, nesting at Shinhama was chiefly two dimensional, *i.e.*, in one plane within 10 feet of the ground, and occurred in a habitat that was botanically homogeneous.

A district of high human population density (10,000 people per square mile) is within 1 to 2 miles of Shinhama at Minami-gyotoku. This village of approximately 1,000 people is separarated from Tokyo only by several miles of rice paddy and the Edo River. Approximately 200 houses, close together and seldom more than one or two deep, lie between a single hard-surfaced road and rice paddies.

The Sagiyama heronry is situated on a long, raised crest of land about 20 miles north of Tokyo (Fig. 2). Immediately surrounding the rookery are dry fields of tea and vegetables, but within one-half mile and 100 feet below are extensive rice paddies where mosquitoes breed. The bird colony itself consists of five farm houses, surrounding yards, animal sheds, fields under cultivation, tea bushes, clusters of bamboo and tall Zelkova trees. Humans, pigs and chickens reside within the colony. A few cows and oxen were also at farms nearby; dogs were rarely seen. For several hundred years, ardeids have bred at Sagiyama and as a result, it is a National Preserve. During 1954–1956 approximately 3,000 to 5,000 ardeids were there during April–October. The egress of birds was gradual each September, but immigration could be quite sudden in April. The birds were Black-crowned Night Herons (BCNH), Plumed Egrets (PE) and Little Egrets (LE) as at Shinhama, but in addition Great Egrets, *Egretta alba*, and Cattle Egrets, *Bubulcus ibis*, were found especially during June and early July. In contrast to Shinhama, nests at Sagiyama were unevenly distributed both horizontally and vertically. Most nests were reached by climbing at least 20 feet into trees. Even nests in bamboo were higher above ground at Sagiyama than at Shinhama. Topographic factors in nesting at Sagiyama not only biased the samples of birds, but played a role in mosquito densities and exposure of birds to mosquitoes throughout the colony (Article III).

During these studies there was a relatively uniform human population density of 3,000 people per square mile for at least 3 miles around Sagiyama. Pigs resided at many farms near Sagiyama and nearby pig dealers estimated the swine population within a 3-mile radius in 1956 to be at least 500 to 1,000 pigs.

EXPERIMENTAL DESIGN

Finally an understanding of the sequence of thought responsible for design of these studies is important for interpretation of results. The philosophy of operation was to integrate field and laboratory efforts. Experiments in nature and the laboratory were designed to complement one another and every attempt was made to interpret field observations promptly and, if indicated, redesign field experiments during the current epidemic season rather than in retrospect during the next interepidemic period. Since the scope and size of habitats are matters of definition and one can usually find microhabitats within macrohabitats, it was realized in 1952 that profitable studies of JE virus ecology required careful definition of study sites and, for practical purposes, initial limitation of hosts and periods of study. Accordingly the Shinhama heronry was chosen as the first habitat to study, mosquitoes and birds were the hosts initially investigated, and spring to fall was the period of study. After data from 1952 and 1953 established certain qualitative facts concerning JE virus at Shinhama, observations were extended to mosquitoes and birds at Sagiyama, to passerine birds in Irumagun, to the Daiganji cormorant colony east of Tokyo, across the bay, and to two colonies of ardeids and cormorants near Nagoya, 150 miles west-southwest of Tokyo (Fig. 1). During 1956 man and domestic pigs were added to the study near Shinhama and Sagiyama. Mosquitoes, birds, man and pigs were observed concurrently in 1956 to determine the relationsip of JE virus in mosquitoes and birds at the heronries to virus in man and domestic animals outside bird colonies. A correlation of these results is presented in Article IX. In essence then, these ecologic studies developed and grew on a yearly basis and yet remained reasonably well integrated despite changes in Army personnel. Their restriction to spring and summer of course minimized opportunities to gain knowledge of the overwintering mechanism of JE virus which may well require entirely different methods of approach and a longer time for resolution.

ACKNOWLEDGEMENTS

Numerous persons, other than the authors, supported these investigations during 1952–1957. The studies were made possible by the Surgeon General, United States Army through his support of research at the 406th Medical General Laboratory under the commands of Colonels R. L. Hullinghorst, R. P. Mason, J. M. Blumberg and M. W. Bayliss. Financially the project was adequately sustained, although personnel rotations at times hampered the program and reduced efficiency. Supplementary personnel for the summers of 1955 and 1956 were provided by Walter Reed Army Institute of Research through the efforts of Drs. J. E. Smadel and G. Edsall. Administratively and professionally many persons assisted in direct and indirect ways. The assistance of each of the following persons and units was indispensible and is deeply appreciated.

Participants from the Department of Virus and Rickettsial Diseases: Drs. S. Hu, L. Kutner, J. Schmidt, and Capt. S. Wade.

S. Anderson, L. Branson, L. D. Baver, Jr., R. Cedeno, J. Dobbins, M. Dobbs, A. Druzd, E. Feldblet, C. Fletcher, T. Fujikura, M. Funkenbusch, K. Hata, R. Highsmith, J. Hunter, S. Iida, F. Kambara, J. Kikkawa, A. Kinoshita, K. Kukino, B. Lynch, F. Mundon, M. Nonga, G. Paul, M. Peck, S. Rodriguez, R. Samuels, M. Shapiro, T. Shibazaki, C. Smith, R. P. Smith, I. Smolin, R. Solarzano, T. Sone, R. Watanuki, A. Wolfe, M. Yorio.

Personnel from the Department of Entomology: W. Suyemoto, S. Toshioka, A. Fulmer and others under the supervision of P. W. Oman, Major MSC (1952–1953), T. E. Blakeslee, Major MSC (1954–1956), and W. P. Murdoch, Capt. MSC (1957).

Personnel from the 207th Medical Detachment, U.S. Army, under commands of M. B. Flemings, Major MSC and H. R. Bullock, 1st Lt. MSC.

The manager of the Shinhama Imperial Duck Refuge, Mr. Adachi, and his assistants. Mr. Tanaka and the owners of the other farms within Sagiyama.

The helicopter service of the Air Wing, First Marine Division and the 8th U.S. Army Aviation

Service and 52nd Medical Detachment (Helicopter Ambulance).

Drs. N. Yokobori, B. Negishi, veterinarians from the Saitama Prefectural Offices, Drs. A. Katsunuma and Kumagai and Mr. Takano from Omiya, Japan, and the many farmers who agreed to bleeding of their pigs.

Personnel from the National Institutes of Health, Tokyo, Japan in association with Dr. M. Kitaoka.

Personnel from the Medical Illustration Department: W. Criss, P. J. Weber and others.

Mr. T. Tanaka, photographer, Kitaurawa, Japan.

The Medical Audio Visual Department, Walter Reed Army Institute of Research, Washington, D. C.

Mrs. L. Katz and Mrs. N. Matilla for secretarial and stenographic efforts.

REFERENCES

1. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z. and McClure, H. E., 1959. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses of birds. *J. Immunol.* **83:** 605–613.
2. Hammon, W. McD., Sather, G. E. and McClure, H. E., 1958. Serologic survey of Japanese B encephalitis virus infection in birds in Japan. *Am. J. Hyg.*, **67:** 118–133.
3. Hotta, S., 1953. Dengue epidemics in Japan, 1942–1945. *J. Trop. Med. and Hyg.*, **56:** 83.
4. Kasahara, S., Ueda, M., Okamoto, Y., Yoshida, S., Hamano, R., and Yamada, R., 1936. Experimental studies on epidemic encephalitis. I. Transmission test of the Japanese encephalitis in 1935 and some characteristics of the infectious agent. *Kitasato Arch. Exp. Med.*, **13:** 48–65.
5. Kawamura, R., Kodama, M., Ito, T., Yasaki, T., and Kobayakawa, Y., 1936. Studies concerning the virus of epidemic encephalitis, Japanese type. *Kitasato Arch. Exp. Med.* **13:** 281–323.
6. Mitamura, T., Kitaoka, M., Mori, K. and Okubo, K., 1938. Isolation of the virus of Japanese epidemic encephalitis from mosquitoes in Okayama City in 1938. *Tokyo Iji Shinshi*, **62:** 820–824 (in Japanese).
7. Sabin, A. B., 1952. Research on dengue during world war II. *Am. J. Trop. Med. Hyg.*, **1:** 30–50.
8. Sonoda, J., 1956. Food and Land: The Deadly Challenge. This is Japan, 1957. Asahi Shimbun Newspaper Publishing Co., Tokyo. **4:** 53–55.
9. Taniguchi, T., Hosokawa, M., and Kuga, S., 1936. A virus isolated in 1935 epidemic of summer encephalitis of Japan. *Jap. J. Exp. Med.*, **14:** 185–196.
10. The World Almanac and Book of Facts for 1959. 1959a, Harry Hansen, Ed., New York World-Telegram, 125 Barclay Street, New York 15, New York, p. 367.
11. The World Almanac and Book of Facts for 1959. 1959b, Harry Hansen, Ed., New York World-Telegram, 125 Barclay Street, New York 15, New York, p. 181.
12. The World Almanac and Book of Facts for 1959. 1959c, Harry Hansen, Ed., New York World-Telegram, 125 Barclay Street, New York 15, New York, p. 191.
13. The World Almanac and Book of Facts for 1959. 1959d, Harry Hansen, Ed., New York World-Telegram, 125 Barclay Street, New York 15, New York, p. 194.
14. The World Almanac and Book of Facts for 1959. 1959e, Harry Hansen, Ed., New York World-Telegram, 125 Barclay Street, New York 15, New York, p. 240.
15. The World Almanac and Book of Facts for 1959. 1959f, Harry Hansen, Ed., New York World-Telegram, 125 Barclay Street, New York 15, New York, p. 269.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN

II. Mosquito Infection

E. L. BUESCHER, W. F. SCHERER, M. Z. ROSENBERG, I. GRESSER, J. L. HARDY, AND H. R. BULLOCK.*

From the Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

INTRODUCTION

Japanese encephalitis (JE) virus was first isolated from mosquitoes collected in Okayama Prefecture, Japan, in 1938.[17, 18] Since then natural mosquito infection has been demonstrated repeatedly in several parts of Japan, usually preceding or accompanying mid-summer human epidemics of JE.[8, 11, 20] From these early studies, it was not possible to determine precisely what species was the important natural vector. Originally, Japanese investigators reported recovery of JE virus from several mosquitoes species,[13, 17, 18] but Barnett found only *C. tritaeniorhynchus* infected in Tokyo during 1950 and 1951.[2] To complicate matters further, a number of laboratory-reared species transmit the virus experimentally.[12, 19, 22] Thus, in 1952 when these studies were begun, it was considered important to examine all prevalent mosquitoes. The investigations were therefore designed to determine the species naturally infected in selected habitats near Tokyo, the time of mosquito infection, the densities of infected mosquitoes and the relationship between total and infected mosquito populations.

Since *C. tritaeniorhynchus* was ultimately shown to be the primary vector of JE virus in this study, certain aspects of its bionomics are summarized to clarify its role as a disseminator of virus. *C. tritaeniorhynchus* is abundant and widely distributed throughout Japan, except in the northern island of Hokkaido.[16] Near Tokyo, adult females are not encountered in significant numbers until mid-June although they first appear in April or May; they occur in largest numbers in late July and in August, and disappear rapidly by early October.[1, 14] Although they are in cities at mid-summer, greater numbers occur in rural habitats, where they breed extensively in paddies.[1] *C. tritaeniorhynchus* attacks large mammals readily, and man to a lesser extent; maximal flight and biting activities occur at dusk and dawn.[1] The mosquito overwinters as inseminated adult females.[7] No conclusive evidence for transovarial transmission of virus has been obtained. In 1956 *C. tritaeniorhynchus* was first colonized in the laboratory[21] and this colonized strain was shown to transmit JE virus experimentally among vertebrates.[9]

MATERIALS AND METHODS

Study sites. Mosquitoes were collected at Shinhama, Sagiyama, Minami-gyotoku, Zama and Tokyo; these habitats are described elsewhere.[24, 25]

Location of mosquito traps and resting stations at study sites. At Shinhama, ground traps were placed in pairs in areas 5 and 6 (Fig. 3 of Article I) during 1952–54, and after 20 July, 1955. Prior to 20 July, 1955 traps were outside the nesting area at the entrance of the Refuge. In 1956 ground traps were in areas 1 to 8. From April–July 1957, three Magoon traps were distributed as follows: a) one in the Refuge, b) one just outside the village at Minami-gyotoku, and c) one in the paddy halfway between the other two.

At Sagiyama in 1955, mosquitoes were collected at dawn from the walls of a cow barn and chicken coop. Ground trap locations in 1956 (9–16) are depicted in Figure 4, Article I. In 1957, six modified Magoon traps were placed in groups of three in the study area and 0.25 mile to the east in a wooded patch adjacent to surrounding paddy. The locations of small ground

* Present addresses: Windham Community Hospital, Willimantic, Connecticut (M. Z. R.); Children's Medical Center, Boston (I. G.); Department of Bacteriology and Immunology, University of Minnesota, Minneapolis (J. L. H.); and 406th Medical General Laboratory, APO 343, San Francisco (H. R. B.).

FIG. 1. Paired bird-baited ground traps at Shinhama. These are located in areas 5 and 6 (Fig. 3, Article I).

FIG. 2. Paired ground traps, Sagiyama, located in areas 9 and 10, and altitude traps, area 21 and 22 (Fig. 4, Article I).

FIG. 3. Ground traps, Sagiyama, located in areas 13 and 14 (Fig. 4, Article I)

FIG. 4. Construction details, typical ground trap, showing internal entrance of mosquito baffle (a), coarse mesh screen confining birds to bottom of trap (b), and lower access hatch (c).

traps at Minami-gyotoku in 1953, of modified Magoon traps at Sagiyama Zama, and Tokyo in 1956 and of altitude traps at Sagiyama in 1956 are described elsewhere.[25]

Designs of mosquito traps. Mosquitoes were collected using two types of traps. Small ground traps (36 x 44 x 40 inches) were constructed of unfinished wood and screen (Figs. 1–4). An en-

tranceway, 5 x 41″, (Figs. 1, 3 and 4) extended the length of each side 10 inches from, and parallel to, the cage bottom, and slanted upward to converge, wedgelike, to an inside opening of 1 x 41 inches, 18 inches from the cage bottom (Fig. 4). In 1952, the interior of the traps was undivided; a single hatch (open door in Fig. 4) at either end admitted head, shoulders and arms of the collector. After it was found in 1952 that chickens ate mosquitoes in traps, birds were confined to the lower half of the cage by ½-inch hardware cloth placed just below the entrance, 18 inches from the bottom (Fig. 4). A third door (lower part of Fig. 4) was constructed at one end to facilitate feeding of bait and cleaning of the trap. The Magoon trap is described and pictured elsewhere.[25]

Bait in mosquito traps. Traps were baited with wild birds, domestic chickens, swine and humans as listed in Table 1 and described elsewhere.[25] Subliming CO_2 ice, an effective attractant for *C. tarsalis* in the U. S. A.,[23] was tried in 1954, but attracted only a few *C. tritaeniorhynchus*. In 1952, 1953 and 1956, only birds without neutralizing (N) antibody to JE virus were used as bait to eliminate antibody in mosquito emulsions, which might neutralize JE virus released upon trituration of infected mosquitoes. In 1956 and 1957, pigs were without antibody until they were infected in August.

Collection and identification of mosquitoes. Numbers of traps, periods, and frequencies of mosquito collections in different years are indicated in Table 1. Mosquitoes were collected by aspirator, transferred to cloth cages and transported promptly to the laboratory where they

TABLE 1

Data concerning collection of mosquitoes, 1952–1957

Study area	Year	Bait for mosquitoes*	Type of trap or collecting station	Number of mosquito traps or resting stations	Collection frequency in days/week	Collection period
Shinhama	1952	BCNH, LE	Small ground trap	3–6	7	13 July–14 Sept
	1953	BCNH, LE, Chick, B Mag, T Spar, D Thru, G Star	Small ground trap	14	7	21 Apr–15 Sept
	1954	BCNH, PE; CO_2	Small ground trap	5	2–3	18 Apr–15 Sept
	1955	BCNH, E	Small ground trap	8	6	20 July–14 Sept
	1956	BCNH	Small ground trap	8	3	30 July–18 Sept
	1957	P	Modified Magoon trap	3	7	1 Apr–30 June
					3	1 July–1 Oct
					1	1 Oct–30 Nov
Minami-gyotoku	1953	BCNH, LE, Chick, B Mag, T Spar, D Thru, G Star	Small ground trap	16	7	21 Apr–15 Sept
	1955	BCNH or PE	Small ground trap	4	6	1 Mar–15 Sept
Sagiyama	1955	Cattle, chickens	Open shed or coop	2	2	25 July–22 Sept
	1956	BCNH	Small ground trap	8	3	27 June–4 Oct
		BCNH	Small altitude trap	6	3	4 July–10 Sept
		P, BCNH, M	Modified Magoon trap	3	3	29 June–3 Oct
	1957	P	Modified Magoon trap	6	7	1 Apr–30 June
					3	1 July–1 Oct
					1	1 Oct–30 Nov
Zama	1956	P, BCNH, M	Modified Magoon trap	3	3	4 July–3 Oct
Tokyo	1956	P, BCNH, M	Modified Magoon trap	3	3	16 June–15 Sept

* BCNH = Black-crowned Night Heron
LE = Little Egret
PE = Plumed Egret
E = Egret, unknown species
Chick = chicken (*Gallus domesticus*)
B Mag = Blue Magpie (*Cyanopica cyaneus*)
D Thru = Dusky Thrush (*Turdus naumanni*)
G Star = Gray Starling (*Spodiopsar cineraceae*)
T Spar = Tree Sparrow (*Passer montanus*)
P = pig
M = man
CO_2 = carbon dioxide (dry ice)

TABLE 2

Variations in methods for isolation of JE virus from mosquitoes

Year	Maximal interval from collection to inoculation	Size of mosquito pool	Diluent for trituration of mosquitoes containing indicated amounts of penicillin and streptomycin	Number, type and routes of inoculation of mice*
1952	6 hours	1-34	Normal saline, pH 7.6, 1000 and 500 u/ml	6–10, 8–10 gm weanlings IC
1953	8–30 hours	1-65	10% rabbit serum† in saline, pH 7.2–7.4, 1000 and 500 u/ml	6–10, 8–10 gm weanlings IC
1954	48 hours	1-70	10% rabbit serum† in saline, pH 7.2–7.4, 1000 and 500 u/ml	6–10, 8–10 gm weanlings IC
1955	6 hrs–6 days‡	1-65	10% rabbit serum† in saline, pH 8, 500 and 100 u/ml	6–10, 8–10 gm weanlings IC
1956				
Ground traps	15 days–4 mo‡	1-65	10% rabbit serum† saline, pH 8, 500 and 100 u/ml	10–20, 1–4 day sucklings from 2 litters, IC and S_Q
Magoon traps	15 days–4 mo‡	1-800	10% rabbit serum† in saline, pH 8, 500 and 100 u/ml	10–20, 1–4 day sucklings from 2 litters, IC and S_Q
1957	15 days–1 mo‡	1-500	10% rabbit serum† in saline, pH 8, 500 and 250 u/ml	10–20, 1–4 day sucklings from 2 litters, IC and S_q

* Albino mice, German swiss strain; IC = intracranial, 0.03 ml for weanlings 0.01 ml for sucklings; S_q = subcutaneous, 0.02 ml for sucklings.

† Rabbit serum was free of JE virus neutralizing antibody upon test in mice.

‡ Mosquitoes not tested for virus within 48 hours of collection were stored at −70° C as whole mosquitoes.

were kept at room temperature in museum jars covered with wet towels until identified, usually within 48 hours. When distance and environmental temperature dictated, transfer cages were refrigerated during transport. Collections were made by trained collectors from the Departments of Entomology or Virus and Rickettsial Diseases, 406th Medical General Laboratory (1952–1957), or the 207th Medical Detachment, U. S. Army (1956–57).

Mosquitoes were anesthetized with chloroform or tobacco smoke (1952–54) or chilled to reduce activity (1955–57), and were identified under 10 to 30× magnification except for several large collections of *C. tritaeniorhynchus* from Magoon traps in June 1956, which were identified by naked eye. Identification was occasionally delayed 24 or 48 hours after collection, either purposefully (1955 resting stations), or for convenience (1956 ground traps).

Isolation of JE virus from mosquitoes. Pools of adult female mosquitoes identified by species, date of collection, and trap source were tested for JE virus (Table 2). Pools from small ground traps contained 1 to 70 mosquitoes and from large Magoon traps, 1 to 800 mosquitoes. The interval between collection and inoculation of mice ranged from 6 hours or less in 1952 to 4 months in 1956. When mosquitoes could not be inoculated within 1 or 2 days of collection, identified mosquitoes were promptly sealed in glass ampules, or in screw-capped vials with adhesive and frozen at −70°C.

Mosquitoes were emulsified in small Ten Broeck grinders or 0.25-ml tuberculin syringes. For each mosquito, 0.04 ml of diluent (Table 2) was added to yield a suspension approximately 10% mosquito tissue by weight. Wherever total volume permitted, suspensions were centrifuged at approximately 500 G for 10 to 12 minutes. From 1952–1955, suspensions were held at 4°C for 1 to 2 hrs before centrifugation. During 1956 and 1957, they were centrifuged immediately. Aliquots of supernatant fluids were sealed in glass ampules for storage on CO_2 ice, and inoculated into locally procured mice as listed in Table 2. When the volume of suspension was less than 0.4 ml (less than ten mosquitoes), the number of mice inoculated approximated the number of mosquitoes in the pool. Mice were observed daily for 12 to 14 days for signs of central nervous system disease. Antigens prepared from brains of sick mice were tested for their ability to fix complement with 4 to 8 units of type-specific antibody to JE, SLE and WEE viruses.[3] Antigens with titers of 1:4 or greater, higher with JE than SLE

antibody, and less than 1:4 with Western equine encephalitis (WEE) antibody, were considered to be JE virus; this procedure, used since 1952 at the 406th MGL, is similar to that described in 1949 for the rapid identification of JE virus by complement-fixation (CF) test.[11]

No effort was made to separate engorged from nonengorged mosquitoes until 1955, when they were tested separately as far as possible. Also in that year, mosquitoes from Sagiyama resting stations containing cows and chickens of unknown immune status were routinely kept alive for 24 hours to permit destruction of N antibody to JE virus which ingested blood might contain. The rationale behind this procedure was mentioned above under *Bait animals*. Each year all tests for virus from mosquitoes were performed in rooms devoted solely to this procedure and by individuals without contact with laboratory strains of JE virus.

RESULTS

Mosquitoes were studied annually to determine: a) species most frequently infected by JE virus; b) times of infection; c) relationships between infected and total mosquito populations as inferred from samples studied*; d) densities of infected mosquitoes; and e) yearly variations in the above particulars.

Mosquitoes at Shinhama and Sagiyama

Species infected by JE virus. Only mosquitoes attracted in large numbers to bird-, pig- and human-baited traps or to resting stations occupied by cows or chickens, were tested for JE virus (Table 1). *C. tritaeniorhynchus* and *C. pipiens* were essentially the only mosquitoes collected from bird-baited traps regardless of the study site (Table 3). In 1956 and 1957 *A. sinensis* was recovered in significant numbers, but only from pig-baited traps.

C. tritaeniorhynchus yielded 298 of 300 strains of JE virus and was therefore the most commonly infected mosquito at Shinhama, and Sagiyama (Table 3). The other two virus strains came from *C. pipiens*. None of 13,960 *A. sinensis* collected at Sagiyama in 1956 or 2286 at Shinhama and Sagiyama in 1957 yielded JE virus upon mouse inoculation.

Viruses were identified as soon as possible after recovery. Since more diseased mice and more potent antigens were obtained after one or two passages of virus, antigens were often made from the second- or third-passage mice, rather than from initially-inoculated mice. Of 126 virus strains from *C. tritaeniorhynchus* during 1952–1956 (Table 3), seven were identified from initially inoculated mice, 105 in second, 13 in third and one at either second or third passage. In 1952 and 1953 JE virus was reisolated from three of five suspensions frozen 3 to 7 months, and in 1956 from nine of ten suspensions frozen for 40 to 69 days. Upon reisolation, the 1956 emulsions titered $10^{1.7}$ to $>10^{4.6}$/0.01 ml intracerebrally in 1 to 4-day-old mice. Thus, all available evidence suggests that isolations of JE virus from *C. tritaeniorhynchus* were technically valid.

The two recoveries of JE virus from *C. pipiens* were also considered technically valid although the recovered viruses may have been from recently ingested viremic blood rather than from infected mosquito tissues. These concepts are discussed further under "Factors Influencing Interpretation of Results." In 1953, a pool of 16 mosquitoes (not engorged as judged by the naked eye) collected August 26th from Shinhama (Table 3) produced disease in two of ten mice on the 7th post inoculation day; one mouse was sacrificed for passage and the other died within 24 hours. The agent was identified in third mouse passage. Sufficient mosquito suspension for reisolation was not available. In 1956, a pool of 143 engorged and nonengorged *C. pipiens* from 26 collections between 29 June and 6 September from a bird-baited Magoon trap at Sagiyama induced disease in two of nine suckling mice on the 2nd and 12th days after inoculation. This agent was identified in second mouse passage. A repeat isolation attempt 18 days after the original was unsuccessful.

Time of mosquito infection. Adequate numbers of mosquitoes were tested in 1952, 1953, 1956 and 1957 to time infection by JE virus. At Shinhama JE virus was recovered from *C. tritaeniorhynchus* only between mid-July and early September (Fig. 5), but at Sagiyama in 1956 infected *C. tritaeniorhynchus* were found from late June to

* Here and elsewhere, the term "mosquito populations" is used with full realization that it is impossible to count numbers of mosquitoes at large. Obviously, mosquitoes cannot be enumerated like birds or pigs. Collections from baited traps at best are samples of unknown proportion to the total population; changes in collection size, however, probably reflect gross variation in the numbers of biting adult females, which are more important than others as potential vectors at any given time.

TABLE 3

Recovery of JE virus from mosquitoes collected near Tokyo late June–early October, 1952–1957

Study area	Year	Trap§	Mosquitoes collected and tested for virus								
			C. tritaeniorhynchus			*C. pipiens*			Other species† & *A. sinensis**		
			Total mosq.	No. of pools	Pools positive	Total mosq.	No. of pools	Pools pos.	Total mosq.	No. of pools	Pools pos.
Shinhama	1952	G	3,039	224	*13*	818	134	0			
	1953	G	4,689	181	*3*	1,140	150	0			
	1954	G	70	22	0	216	56	0	5	4	0
	1955	G	993	118	0	1,868	171	*1*	29	5	0
	1956	G	3,842	123	*9*	385	16	0	8	5	0
	1957	M	44,019	245	*68*				402*	17*	0*
Minami-gyotoku	1953	G	1,896	261	0	20,494	500‡	0			
	1955	G	100	39	0	9,984	412	0	41	15	0
Sagiyama	1955	RS	276	19	*4*	25	9	0	45	19	0
	1956	G	22,974	519	*22*	528	23	0	187	40	0
	1956	M	98,870	279	*66*	243	5	*1*	741	22	0
									13,960*	30*	0*
	1957	M	50,613	347	*113*				1,884*	27*	0*
Zama	1956	M	2,524	15	7	675	6	0	561	13	0
									573*	3*	0*
Tokyo	1956	M	196	8	*2*	620	8	0	73	7	0
Totals			234,101	2,400	307	36,996	1,490	2			

* Numbers of *A. sinensis* marked with asterisk.

§ G = bird-baited small ground traps; M = bird-, pig- and human-baited Magoon traps in 1956 and pig-baited in 1957; RS = resting stations (cows and chickens).

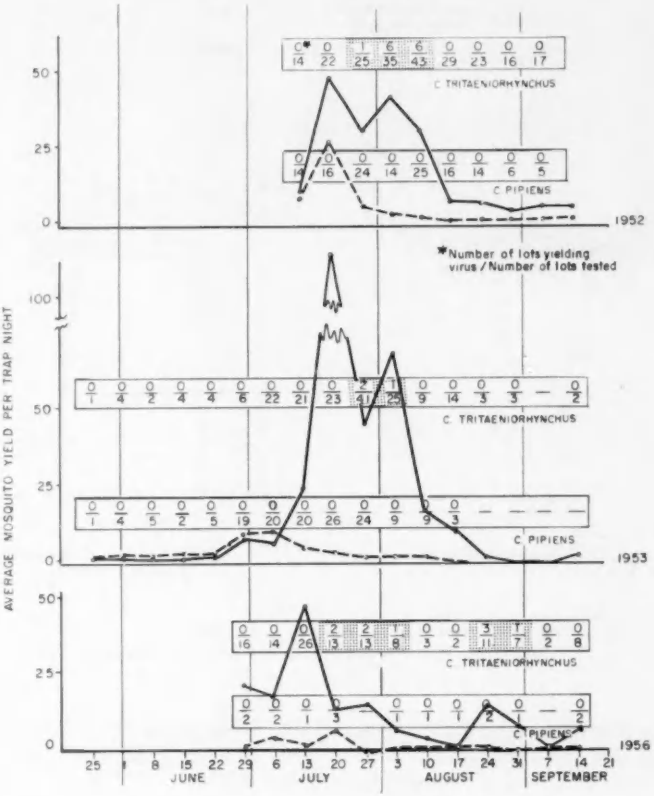
† Includes: *Culex bitaeniorhynchus, Aedes vexans nipponi, Culex orientalis, Aedes albopictus, Culex vorax and Aedes nipponicus.*

‡ Approximate numbers; collected in April, May & June, 1953.

late September (Figs. 6 and 7). In 1957 mosquito infection was first detected in mid-July at Shinhama and in early August at Sagiyama, and continued into early October.

It is of interest to note that in 1956 peak mosquito populations were larger and occurred earlier at Sagiyama, than at Shinhama and, perhaps as a consequence, infected mosquitoes were found several weeks earlier at Sagiyama than at Shinhama. These temporal differences were real because collection methods were identical at both locations and were made by the same persons on alternate days. Thus, from place to place in the Kanto plain, time of occurrence of infected mosquitoes can vary several weeks in onset, and might influence the variation in time of occurrence of human and equine disease.[6]

Relationship of infected to total C. tritaeniorhynchus *populations.* Infected mosquitoes were obtained in largest numbers during the periods of total population decline at Shinhama (Fig. 5) and Sagiyama (Figs. 6 and 7). Only a few isolates were obtained in late June and early July 1956, at the mosquito population peak. Average mosquito yields per heron trap-night were selected to represent total mosquito populations because BCNH-baited traps collected more *C. tritaeniorhynchus* than those containing other bird species[25] and because BCNH comprise a numerically important segment of the vertebrate population at Shinhama. The numbers of virus strains recovered were also independent of total mosquito population. For example, 13 strains were recovered in 1952 although the total *C. tritaeniorhynchus* population appeared to be less than in 1953 when only three strains were isolated by identical laboratory methods (Fig. 5)

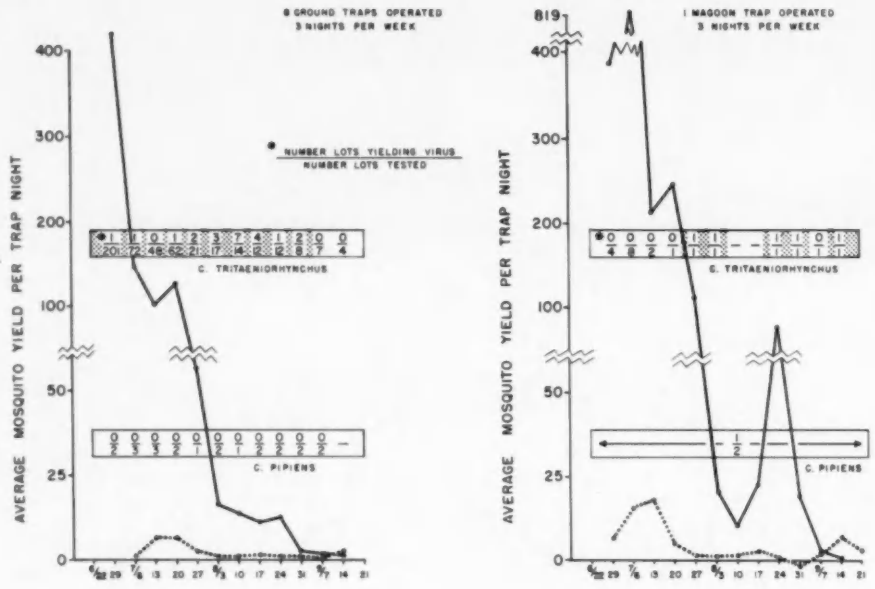


TIME AND INCIDENCE OF MOSQUITO INFECTION WITH JE VIRUS; HERON BAITED GROUND TRAPS SHIN HAMA, JAPAN

FIG. 5

*Density of transmitting mosquitoes.** The density of transmitting mosquitoes (*the absolute number* in a *specified space* at a *given time*) obviously is a critical measure of a vector's potential to disseminate virus to vertebrates. Density of transmitting mosquitoes cannot be measured directly since the only available method for studying natural mosquito infection *identifies infected, but not necessarily transmitting mosquitoes.* For example, the virus in certain *C. tritaeniorhynchus* may have been only recently ingested, and not as yet transmissible by bite.[9, 10] Yet, since most infected mosquitoes eventually transmit within 2 to 3 weeks after infection, density of infected mosquitoes is probably proportional to density of transmitting mosquitoes. However, the real problem lies in the enumeration of infected mosquitoes. One cannot simply calculate the absolute number of infected mosquitoes in a study site from the frequency of infected mosquito pools because the latter does not necessarily represent the actual frequency of *individual* infected mosquitoes, and because the total mosquito population cannot be measured accurately. Determination of the prevalence of infected mosquitoes would require testing hundreds to thousands of individual mosquitoes for

* In this description, *frequency* is defined as the "ratio of actual to possible occurrences of an event"; *prevalence*, as the "proportion of the population which exhibits a phenomenon *at* a given time;" and incidence, the "rate of occurrence of a phenomenon *during* a given period of time."


8 GROUND TRAPS OPERATED 3 NIGHTS PER WEEK
1 MAGOON TRAP OPERATED 3 NIGHTS PER WEEK
NUMBER LOTS YIELDING VIRUS / NUMBER LOTS TESTED
AVERAGE MOSQUITO YIELD PER TRAP NIGHT
C. TRITAENIORHYNCHUS
C. PIPIENS
819

TIME AND INCIDENCE OF MOSQUITO INFECTION WITH JE VIRUS
HERON BAITED GROUND AND MAGOON TRAPS, SAGIYAMA, JAPAN - 1956

FIG. 6

virus, since the prevalence of infection may well be less than .01. Yet with total populations of a million or more,† hundreds to thousands of infected mosquitoes might exist when the infection frequency is less than .01. Thus, in analyzing results of mosquito infection studies, and attempting to relate them to virus ecology and vertebrate infection, one faces the dilemma of being unable to measure the critical factor in the vector's potential to disseminate virus (*viz.*, density of transmitting mosquitoes) and is forced to attempt interpretations from crudely measured infection frequencies determined with pooled mosquitoes. The observations resulting from such an attempt in these studies are as follows:

1. Infection frequency ranged from 2 to 50% (Table 4), assuming that only one mosquito in each pool contained virus, and that traps were not selecting infected mosquitoes.

2. Frequency of detection of infected *C. tritaeniorhynchus* increased progressively during summer, remained high into September, and decreased thereafter in the samples tested (see ratios of infected mosquito pools to totals tested for single years; Figures 5 to 7). The decrease in infection frequency in the sample, however, does not necessarily mean that the infection frequency of the natural population changed markedly, since there is no evidence that infected mosquitoes have a shorter than usual life span; rather, it probably resulted from smaller collections. On the other hand, infected mosquito density obviously waned in direct proportion to the decline in total population.

3. Frequencies of detection of infected mosquitoes during June–September varied from

† Mosquitoes inhabiting Shinhama and Sagiyama are bred in the vast surrounding paddy. The recovery of 10^3 to 10^4 *C. tritaeniorhynchus* daily, or every other day would have depleted a total population of 10^5 or less within 14 to 21 days, once emergence had reached its peak. Since at Sagiyama in 1956 more than 10^3 *C. tritaeniorhynchus* were collected every other day for at least 8 weeks (Figs. 6 and 7), the total population was obviously not being depleted at this rate. Therefore it may be inferred that the total population of the habitat and its surrounding paddy was of the order of 10^6 or more, and that collections of 10^2 to 10^4 mosquitoes probably represented .01 to 1.0% of the total.

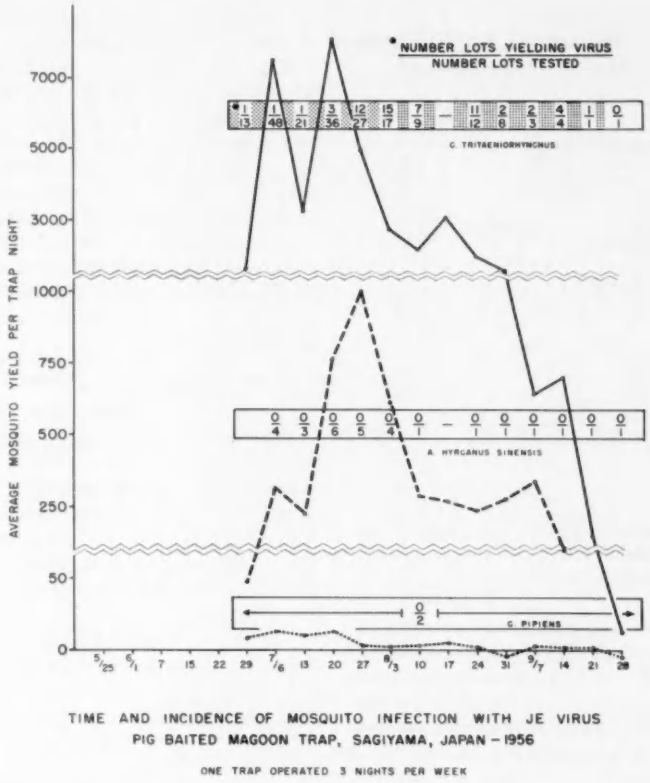


FIG. 7

year to year at the same study site. For example, 13 isolates were obtained from 3,039 *C. tritaeniorhynchus* at Shinhama in 1952 (1 virus strain per 233 mosquitoes), but in 1953, only three were found in 4,689 mosquitoes (1 in 1563) (Table 3). Yet densities of infected mosquitoes probably were comparable in these 2 years since the total population appears to have been greater in 1953 than in 1952 (Fig. 5). Such variations appear to be real since, in 1952 and 1953, traps were in the same location in Shinhama,[25] and most of the mosquitoes were collected during the same time interval.

4. Frequencies of detection of infected mosquitoes could be roughly comparable at different study sites during the same year. For example, during 1956, nine virus strains (each representing at least one infected mosquito per pool) were recovered from 3,842 *C. tritaeniorhynchus* at Shinhama (1 in 427) whereas 22 viruses were obtained from 22,974 mosquitoes (1 in 1044) collected in the same types of traps (BCNH-baited, ground) at Sagiyama (Table 3). Similar ratios for Shinhama and Sagiyama pig-baited traps in 1957 were one in 647 and one in 448. However, since collections and, therefore, probably total populations, varied in size, densities of infected and transmitting mosquitoes probably differed between study sites.

Early season mosquito infection. No infected *C. tritaeniorhynchus* or other numerically important species were recovered during the spring of 1953 at Shinhama and Minami-gyotoku or of 1957 at Shinhama and Sagiyama (Table 5). In 1953, two species, *C. tritaeniorhynchus* and *C. pipiens*, were collected with regularity from bird-baited traps during April, May and June, but no virus was recovered from approximately 200 *C. tritaeniorhyn-*

TABLE 4

Numbers of C. tritaeniorhynchus *in samples containing at least one infected mosquito*

Location	Year	Trap§	No. of *C. tritaeniorhynchus* in pools yielding virus						
			2–10	11–20	21–50	51–100	101–200	201–500	501–800
Shin-	1952	G		3	10				
hama	1953	G			3				
	1956	G	1*		8				
	1957	M			2	2	58	6	
Sagi-	1955	RS		2	2				
yama	1956	G	2†	2	16	2			
	1956	M			3	2	2	57	2
	1957	M		1	5	17	76	14	
Zama	1956	M				2	2	3	
Tokyo	1956	M			2				
Totals			3	8	51	25	138	80	2

* Virus recovered from 6 mosquitoes.
† Virus recovered from 2 and 4 mosquitoes.
§ See footnote§ Table 3.

chus or 20,000 *C. pipiens*. Similarly, JE virus was not encountered in approximately 45,000 *C. tritaeniorhynchus* taken from pig-baited traps at Shinhama and Sagiyama during April–June 1957, even though some were kept in an insectary under simulated summer environments for 2 weeks before test. The numbers so tested were 84 from Shinhama and 2 from Sagiyama in April, 310 and 276 in May and 139 and 222 from each location in June. Other mosquitoes from pig-baited traps (*A. vexans*, 21,000; *C. pipiens*, 6,000; *A. sinensis*, 2,000) unfortunately were not tested directly for JE virus by animal inoculation during April–June 1957. However, none of the nine pigs in mosquito traps showed serologic evidence of infection with JE virus before mid-July, although they promptly developed antibodies after infected *C. tritaeniorhynchus* were recovered from the traps.

Mosquitoes at Zama and Tokyo

In 1956, infection of mosquitoes in representative habitats of Tokyo and Zama[25] was studied simultaneously with Shinhama and Sagiyama (Table 1). Fewer mosquitoes were collected at Zama and Tokyo than at the other study sites. Only *C. tritaeniorhynchus*, and not *C. pipiens* or *A. sinensis*, was infected (Table 3). Time of initial mosquito infection, as determined by both isolation of JE virus and infection of sentinel animals, was early July for Zama and early August for Tokyo. Because only small numbers of *C. tritaeniorhynchus* were collected, the duration of mosquito infection in these two sites was not accurately determined. However, infected *C. tritaeniorhynchus* were recovered as late as mid-September at Zama, and late August at Tokyo. Thus, it would appear that mosquito infection at Zama and Tokyo was coincident with that at Shinhama and Sagiyama with respect to species infected, time of initial infection and duration of the infection period.

Factors Influencing Interpretation of Results

Interpretation of these studies depends upon: 1) the thoroughness with which mosquitoes were collected from traps, and validities of mosquito identifications and virus isolations; 2) the significance of virus or the failure to find it in a mosquito emulsion; 3) the source of virus for mosquitoes; and 4) the accuracy with which the collection methods reflect the total mosquito populations, and their variations.

Validity of mosquito identification and virus isolations. Competence of mosquito collection and identification teams has already been mentioned under *Methods*. In 1952, 1953, and 1955, isolations of virus from mosquitoes were made in rooms from which laboratory strains of virus were excluded, until each day's mosquito suspensions were inoculated. In 1956 and 1957 when space permitted, mosquito isolation work was done in a room reserved only for this purpose. Each year this work was performed by one or two persons without other duties. Weanling Swiss mice were reared on farms near Tokyo, and during 1952 and 1953, in screened quarters, under supervision of laboratory personnel. In 1954 and 1955, this supervision was not rigidly enforced, but since only four virus recoveries were made, the possibility of isolating virus from weanling mice "spontaneously infected" in nature[15] was essentially irrelevant. That such a possibility was unlikely for the study as a whole is supported by the following observations: a) JE virus was never encountered in uninoculated mice. b) Weanling mice in the field attract, and are bitten by, the vector *C. tritaeniorhynchus* only rarely (Fig. 10, Article 3 of this series). c) For practical purposes, isolation of virus was confined to *C. tritaeniorhynchus*, and the times of

TABLE 5

Failure to detect virus in mosquitoes during spring, 1953 and 1957

Mosquito species	Year	Location	Mosquitoes tested for JE virus by month								
			April			May			June		
			Total mosq.	No. of pools	Pools pos.	Total mosq.	No. of pools	Pools pos.	Total mosq.	No. of pools	Pools pos.
C. tritaeniorhynchus	1953	Shinhama	0			2	2	0	31	18	0
		Minami-gyotoku	0			12	7	0	153	48	0
	1957	Shinhama	265	11	0	728	33		17,642	144	0
		Sagiyama	4	2	0	633	25	0	26,053	211	0
C. pipiens	1953	Shinhama	9	3	0	18	13	0	316	50*	0
		Minami-gyotoku	710	75*	0	3,826	125*	0	15,615	250*	0

* Approximate numbers.

infection were similar each year, despite the fact that both infected and noninfected species were tested at random, and simultaneously. d) Virus was reisolated from several mosquito suspensions in 1952 and 1956. e) The frequency of virus isolations was far greater than that explained by natural mouse infection.

Significance of recovery of virus from emulsions. The finding of JE virus in an emulsion of *C. tritaeniorhynchus* has two possible explanations: a) that virus has multiplied in the mosquito, and b) that virus simply was recently ingested from a viremic animal. Two procedures were employed in 1955 in an attempt to differentiate between these possibilities: 1) nonengorged mosquitoes were tested separately from engorged; and 2) mosquitoes were held alive in the laboratory for 24 hours before testing assuming that free, intestinal virus would be digested or eclipsed within a day of ingestion. Neither procedure answered the question, since so little virus was isolated in 1955 (Table 3). Further, virus ingested by colonized *C. tritaeniorhynchus* is still detectable 1 day after feeding.[10] However, since up to 94% of colonized *C. tritaeniorhynchus* ingesting 3.0 or more LD_{50} of virus became infected, and since 43 to 92% of infected mosquitoes will transmit 15 or more days after infection, wild caught *C. tritaeniorhynchus* with recently ingested virus are probably as important ecologically as these actually infected, since both are eventually capable of transmission (being infective). However, it is probable that collections from animal-baited traps consisted of fewer recently infected mosquitoes than the natural populations, since a recently fed mosquito was perhaps less likely to enter baited traps than one which had fed 10 or more days earlier.

The failure to find virus in a mosquito suspension may mean: a) that virus was absent; or b) that it was present, but below detectable levels. The methods used in this study for virus isolation probably did not detect every infected mosquito. By the same methods used here, virus was not detectable in single *C. tritaeniorhynchus* 2 to 4 days after ingestion.[10] However, testing pools of 20 to 500 mosquitoes each may have diluted virus below detectable concentrations. Thus, a single mosquito containing 100 LD_{50} could be suspended along with 499 uninfected mosquitoes, in a volume of 20 ml (0.04 ml/mosquito). In this circumstance the 0.03 ml inoculated into a single weanling mouse would contain but 0.15 LD_{50} (0.03 ml $\times$ 100 LD_{50}/20 ml), an amount of virus not regularly detectable. Only in mosquitoes in which virus had multiplied for 5 to 10 days to levels of $10^{3.5}$ LD_{50} would it be detectable after such dilution.

Source of virus for mosquitoes. The usual source of virus for mosquitoes collected from animal-baited traps is considered to be animals outside traps rather than those used as bait. Since viremia in trap animals lasts only 2–5 days[4, 26] it is probable that no more than a few days' collections of infected mosquitoes represented virus from animal bait.

Adequacy of sampling of mosquito populations. Throughout these studies, mosquito collections from animal-baited traps were recognized as being only rough approximations of actual populations in nearby environments, and their changes with time. To check the mosquito collections from bird-baited traps, a light trap was operated simultaneously from 13 July–28 September, 1956, at Sagiyama. The yields of *C. tritaeniorhynchus* were greatest in July and decreased sharply in early August in parallel with collections from BCNH-baited ground traps (Fig. 6.). Thus, two collection methods suggested that the *C. tritaeniorhynchus* population at Sagiyama had diminished in size by early August (Fig. 6); yet collections from nearby pig-baited traps continued to be large throughout August and early September (Fig. 7). This discrepancy between collections by different methods cannot be explained, but might mean: a) that below critical levels, pig-baited traps are more sensitive indicators of *C. tritaeniorhynchus* populations than BCNH-baited ones; b) that *C. tritaeniorhynchus* naturally exist in heterogenous groups with different animal tropisms, and with chronologically different population curves (considered unlikely); or c) that the tropism of *C. tritaeniorhynchus* for birds, but not for pigs, changes during the summer. Regardless of the explanation, however, such data emphasize the uncertainties that accompany use of a single trapping method in attempting to evaluate total mosquito populations.

DISCUSSION

Repeated linear investigation of the mosquitoes of several limited habitats near Tokyo established that infection of mosquitoes with JE virus was a regular late summer phenomenon, limited essentially to *C. tritaeniorhynchus*, and coincident with ardeid infection at Shinhama, and ardeid and porcine infection at Sagiyama.[5, 27] Thus, 307 of 309 virus strains were recovered from *C. tritaeniorhynchus* during the period (mid-July to mid-September) when dissemination of JE virus among birds and pigs of the study area occurred. In both study areas, the frequency of infected mosquitoes increased during this period to reach maximal levels of one infected mosquito in 2 to 20 (5–50%) during late August, *after* the peak populations of *C. tritaeniorhynchus* had been attained. Infection frequencies also varied from year to year in a given place. Thus, at Shinhama, infection frequencies encountered in 1952, 1956 and 1957 were higher than those of 1953. Accurate determinations of the *total* mosquito population and its week to week variation are impossible to make; these frequencies therefore, while offering indices of mosquito infection, can be used to obtain only the crudest estimates of the density of infected mosquitoes. Within the limits of the present methods, however, it would appear that there were no significant differences in infected-mosquito densities between Shinhama and Minami-gyotoku in 1953 or among Shinhama, Sagiyama, Zama and Tokyo in 1956. Infection of *C. pipiens* was so rare as to appear ecologically unimportant; possibly the two isolates from *C. pipiens* represented only recently-ingested virus from viremic birds. *A. sinensis* was not found to be infected, even though its tropism for pigs, which are an excellent source for JE virus in nature,[27] was as great or greater than that of *C. tritaeniorhynchus*.[25]

No evidence of mosquito infection was obtained during the spring (April–June), nor was it determined how the infection cycle is initiated each summer. Virus may overwinter in hibernating female *C. tritaeniorhynchus* to emerge again in spring; but, if this occurs, the amount of overwintering virus must be minute since hibernating females are found only in exceedingly small numbers during the winter. In the summer, infected *C. tritaeniorhynchus* occurred in largest numbers after the peak total mosquito population when the total population was declining. This temporal dissociation between infected and total populations of *C. tritaeniorhynchus* suggests that infection occurs from an extra-mosquito source of virus, *e.g.*, viremic birds and pigs[6, 27] and not as a simple function of *C. tritaeniorhynchus* reproduction. If the virus were passed from mosquito to mosquito transovarially in nature, one would expect the numbers of infected *C. tritaeniorhynchus* to increase simultaneously with the total population. This conclusion is supported by our inability to recover JE virus from *C. tritaeniorhynchus* eggs, larvae and pupae collected in nature during 1952 at Shinhama, and by Barnett's failure to find virus in adult males in 1950 (Barnett, personal communication).

The reason that *A. sinensis* did not yield detectable virus despite its presence in large numbers in pig traps at heronries is possibly because it bites birds infrequently, and thus did not acquire virus readily at locations where birds are

the major source of virus for mosquitoes. Certainly our data do not exclude *A. sinensis* as a vector outside the Sagiyama heronry, although Barnett failed to find JE virus in 1,208 *A. sinensis* from Tokyo and 8,817 from Okayama in 1949 and in 5232 from Tokyo in 1950.[2] Further transmission experiments under controlled laboratory conditions would certainly seem justified to extend the observations of Mitamura *et al.*, who showed that *A. sinensis* can transmit JE virus after artificial infection in the laboratory,[19] and to clarify the factors apparently limiting natural transmission of virus by this mosquito.

Knowledge of the temporal discrepancy between total and infected populations of vector mosquitoes makes it clear why total population curves have been difficult to correlate with human epidemics. The fact that total *C. tritaeniorhynchus* population peaks precede human epidemics by up to 4 weeks[6] now becomes irrelevant when one realizes that infected mosquitoes occur several weeks after the total population increase and, indeed, do correspond in time with human epidemics,[28] equine epizootics,[6] and avian[5] and swine[27] infection.

SUMMARY

Linear study of mosquitoes in several habitats near Tokyo between 1952 and 1957 established that the only species consistently infected with JE virus naturally was *C. tritaeniorhynchus*. Mosquito infection was a regular summer phenomenon limited to late June–early October. Density of infected mosquitoes at a study site is considered the important ecologic concept rather than frequency of infected mosquitoes in samples tested. Although infection frequencies varied yearly in one area (Shinhama) and were similar from place to place in single years, densities of infected mosquitoes may have been just the opposite because differences in total populations overcompensated for changes in infection frequencies. Natural frequencies of infected mosquitoes increased progressively during the summer to reach maxima of one in 2 to 20. Density of infected mosquitoes increased concomitantly with frequency until peak frequencies were reached and total populations declined enough to reduce the density of infected mosquitoes at a study site. Time of *C. tritaeniorhynchus* infection was independent of total population and the incidence of mosquito infection increased while the total population declined. It is therefore particularly important to emphasize that there is no direct, chronologic relationship between peak vector populations, and epidemic or epizootic infection. However, infection of vertebrates occurs coincident with, and as a consequence of, mosquito infection and is related directly to the time when *maximal density of infected mosquitoes* occurs in a specific area.

REFERENCES

1. Barnett, H. C., and others, 1950. Insect studies in relation to Japanese B encephalitis. Annual Professional Report, 406th Medical General Laboratory, U. S. Army: 160–185.
2. Barnett, H. C., and others, 1951. Virus isolation studies on mosquito material collected in 1950 in Tokyo. Annual Professional Report, 406th Medical General Laboratory, U. S. Army: 231–240.
3. Buescher, E. L., Scherer, W. F., Grossberg, S. E., Chanock, R. M., and Philpot, V. B., Jr., 1959a. Immunologic studies of Japanese encephalitis virus in Japan. I. Antibody responses following overt infection of man. *J. immunol.*, **83:** 582–593.
4. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., and McClure, H. E., 1959b. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses in birds. *J. Immunol.*, **83:** 605–613.
5. Buescher, E. L., Scherer, W. F., McClure, H. E., Moyer, J. T., Rosenberg, M. Z., Yoshii, M., and Okada, Y., 1959c. Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. *Am. J. Trop. Med. & Hyg.*, **8:** 678–688.
6. Buescher, E. L., and Scherer, W. F., 1959d. Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemiologic correlations and conclusions. *Am. J. Trop. Med. & Hyg.*, **8:** 719–722.
7. Bullock, H. R., Murdock, W. P., and others, 1957. Bionomics of *Culex tritaeniorhynchus*; overwintering studies. Annual Professional Report, 406th Medical General Laboratory, U. S. Army: 61–69.
8. Fukuzumi, T., Ito, H., Tsubaki, S., Ozawa, K., Toda, M., Kumano, T., Homma, S., Obata, Y., Kubono, M., Owada, H., Takahashi, K., Sho, S., Sagawa, T., Shimada, H., and Sasaki, A., 1949. Studies on Japanese B encephalitis in 1948. Commission for the Research of Japanese B encephalitis report, 1948–1949: 91–102. (in Japanese)
9. Gresser, I., Hardy, J. L., Hu, S. M. K., and Scherer, W. F., 1958a. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* from infected pigs and chicks to susceptible pigs and birds. *Am. J. Trop. Med. and Hyg.*, **7:** 365–373.
10. Gresser, I., Hardy, J. L., Hu, S. M. K., and Scherer, W. F., 1958b. The growth curve of Japanese encephalitis virus in the vector mosquito of Japan, *Culex tritaeniorhynchus*. *Japan J. Exp. Med.*, **28:** 243–248.
11. Hammon, W. McD., Tigertt, W. D., Sather,

G., AND SCHENKER, H., 1949a. Isolations of Japanese B encephalitis virus from naturally infected *Culex tritaeniorhynchus* collected in Japan. *Am. J. Hyg.*, **50:** 51–56.

12. HAMMON, W. McD., REES, D. M., CASALS, J. AND MEIKLEJOHN, G., 1949b. Experimental transmission of Japanese B encephalitis virus by *Culex tritaeniorhynchus* and *Culex pipiens pallens*, suspected natural vectors. *Am. J. Hyg.*, **50:** 46–50.
13. KITAOKA, M., MIURA, T., NAKAMURA, K., AND BABA, S., 1949. Isolation of the virus of Japanese B encephalitis from mosquitoes collected in the Tokyo area in the summer of 1948. Commission for the Research of Japanese B Encephalitis. 1948–1949; 130–132.
14. KITAOKA, M., MIURA, T., AND OGATA, T., 1950a. Seasonal occurrence of mosquitoes in nature in Tokyo in 1948 and 1949. Zoophilia of each species of mosquito. *Jap. Med. J.*, **3:** 331–347.
15. KITAOKA, M., OKUBO, K., KUMA, S., AND BABA, S., 1950b. Isolation of Japanese B encephalitis virus and unknown filtrable viruses from the mice bought from the mouse dealer in the summer of 1948. *Nihon Noen*, 1950: 247–248. (in Japanese)
16. LACASSE, W. J., AND YAMAGUTI, S., 1950. *Mosquito fauna of Japan and Korea.* Published by Surgeon, 8th U. S. Army, Japan, p. 235.
17. MITAMURA, T., KITAOKA, M., MORI, K., AND OKUBO, K., 1938. Isolation of the virus of Japanese epidemic encephalitis from mosquitoes caught in nature. *Tokyo Iji Shinshi*, **62:** 820–824. (in Japanese)
18. MITAMURA, T., KITAOKA, M., WATANABE, S., TENJIN, S., HOSOI, T., SAKI, O., NAGAHATA, I., JO, K., AND SHIMIZU, M., 1939. Seasonal variation in number of mosquitoes and in presence of virus in mosquitoes in Okayama City in 1938. *Tokyo Iji Shinshi*, **63:** 1980–1983. (in Japanese)
19. MITAMURA, T., MORI, K., KITAOKA, M., AND TENJIN, S., 1947. Experimental transmission of Japanese B encephalitis by artificially infected mosquitoes. *Tokyo Iji Shinshi*, **3076:** 812–819. (in Japanese)
20. MITAMURA, T., KITAOKA, M., WATANOBE, S., IWASKI, T., TENJIN, S., ISHIKAWA, I., JO, K., YOSHIDA, H., YOSHIMATSU, D., WARABIOKA, K., SHIMIZU, H. AND WATANABE, T., 1950. Seasonal variations in the number of mosquitoes and in the presence of virus in mosquitoes in Okayama City in 1949. *Nihon Igaku Oyobi Kenko Hoken*, **1:** 743–745. (in Japanese)
21. NEWSON, H. D., BLAKESLEE, T. E., TOSHIOKA, S., SAKAI, M., WHEELER, C. M., SHIMADA, T., AND AKIYAMA, J., 1956. A preliminary report on the laboratory colonization of the mosquito *Culex tritaeniorhynchus* giles. *Mosq. News*, **16:** 282.
22. REEVES, W. C., AND HAMMON, W. McD., 1946. Laboratory transmission of Japanese B encephalitis virus by seven species (three genera) of North American mosquitoes. *J. Exp. Med.*, **83:** 185–194.
23. REEVES, W. C. 1953. Quantitative field studies on a carbon dioxide chemotropism of mosquitoes. *Am. J Trop. Med. and Hyg.*, **2:** 325–331.
24. SCHERER, W. F., AND BUESCHER, E. L., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. *Am. J. Trop. Med. & Hyg.*, **8:** 644–650.
25. SCHERER, W. F., BUESCHER, E. L., FLEMINGS, M. B., NOGUCHI, A., AND SCANLON, J., 1959b. Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors. Zootropism and vertical flight of *Culex tritaeniorhynchus* with observations on variations in collections from animal baited traps in different habitats. *Am. J. Trop. Med. & Hyg.*, **8:** 665–677.
26. SCHERER, W. F., MOYER, J. T., AND IZUMI, T., 1959c. Immunologic studies of Japanese encephalitis virus in Japan. V. Maternal antibodies, antibody responses and viremia following infection of swine. *J. Immunol.*, **83:** 620–626.
27. SCHERER, W. F., MOYER, J. T., AND IZUMI, T., 1959d. Ecologic studies of Japanese encephalitis virus in Japan. VI. Swine infection. *Am. J. Trop. Med. & Hyg.*, **8:** 698–706.
28. SCHERER, W. F., KITAOKA, M., OKUNO, T., AND OGATA, T., 1959e. Ecologic studies of Japanese encephalitis virus in Japan. VII. Human infection. *Am. J. Trop. Med. & Hyg.*, **8:** 707–715.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN

III. Mosquito Factors. Zootropism and Vertical Flight of *Culex tritaeniorhynchus* with Observations on Variations in Collections from Animal-baited Traps in Different Habitats

W. F. SCHERER, E. L. BUESCHER, M. B. FLEMINGS, A. NOGUCHI, and J. SCANLON*

From the Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

INTRODUCTION

Behavioral patterns of vector mosquitoes are well recognized factors influencing dissemination of arthropod-borne viruses, a notable example being the behaviors of *Aedes aegypti* and *Haemogogus spp.* in epidemic and jungle yellow fever.[16] After studies at Shinhama in 1952 suggested that *C. tritaeniorhynchus* was the only important vector of Japanese encephalitis (JE) virus,[3] it seemed pertinent to determine how behavior of this mosquito influenced dissemination of JE virus. This article records observations from three investigations: 1) a study of the tropism† of *C. tritaeniorhynchus* for 7 different bird species; 2) a comparison of Black-crowned Night Herons, pigs and man as attractors of mosquitoes; and 3) an examination of vertical flight range to learn whether differences in nest altitude affect transmission of virus between mosquitoes and birds.

MATERIALS AND METHODS

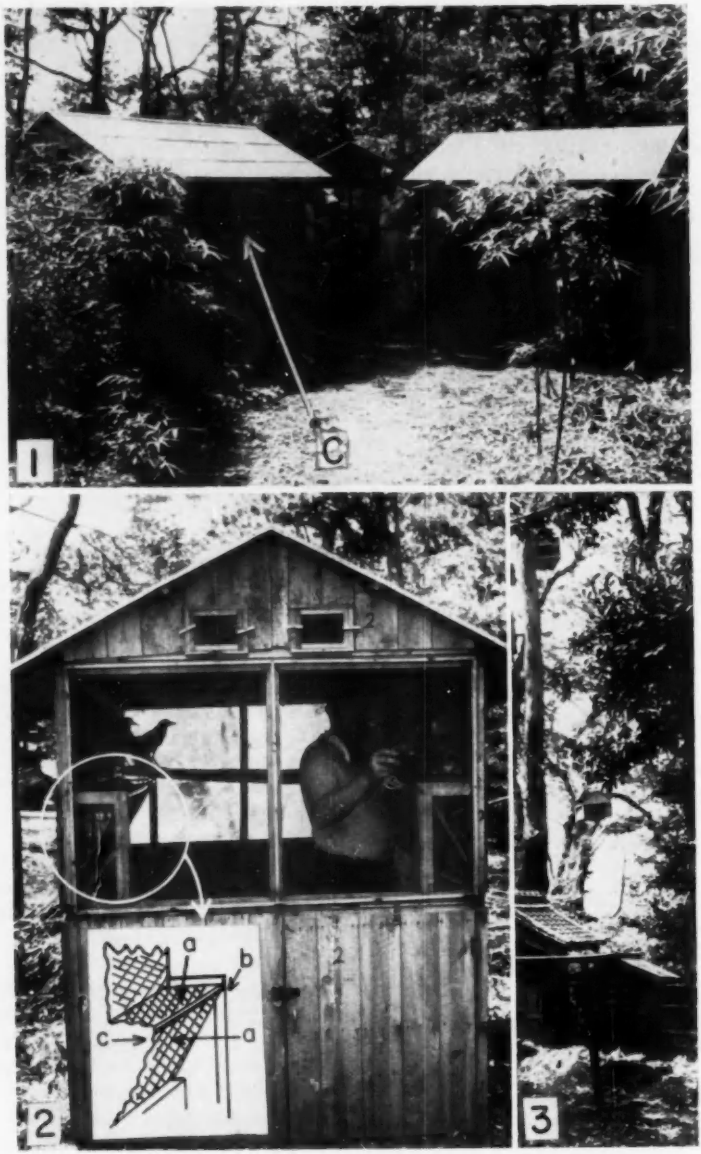
Study sites. In 1953, mosquito behavior was studied at Shinhama and the nearby village of Minami-gyotoku.[14] Three sites, 20 to 30 miles apart, were employed in 1956 (Sagiyama, Figs. 1-3;[14] Zama and Tokyo). A shrine in a wooded area amidst rice farms at Zama (Figs. 4 and 5) was chosen because, except for a heronry, it was ecologically similar to Sagiyama. Observations of urban dwelling mosquitoes were made in the Setagaya district of Tokyo because it had a dense human population of 14,000 per square mile and was convenient to the collaborating 207th Medical Detachment Laboratory. Traps were at a shrine (Figs. 6 and 7) surrounded on three sides by houses and apartments and, on the fourth side, by a street, small stream, and within 100 yards, dense housing.

Location of mosquito traps at study sites. In 1953 at Shinhama, a group of traps was located on the ground in areas 5 and 6 (Fig. 3, Article I). Paired traps were employed for each species of bird. At Minami-gyotoku, duplicate traps with birds of each species were widely separated and placed at random behind houses on each side of the main street.[14] In 1956, modified Magoon traps[2, 11] were located at Sagiyama as depicted in Figure 4 of Article I and were arranged at each study site as spokes of a wheel 120 degrees apart (Figs. 1, 5 and 6). Six traps were suspended in trees (Fig. 3) between 24 and 50 feet above ground during July and August 1956 at Sagiyama. Single traps were at 24 to 27 feet and at 43 to 50 feet in each of two trees (areas 17, 18, 21, 22, Fig. 4, Article I) at 43 to 50 feet in a third tree (area 19), and at 33 feet in a fourth tree (area 20).

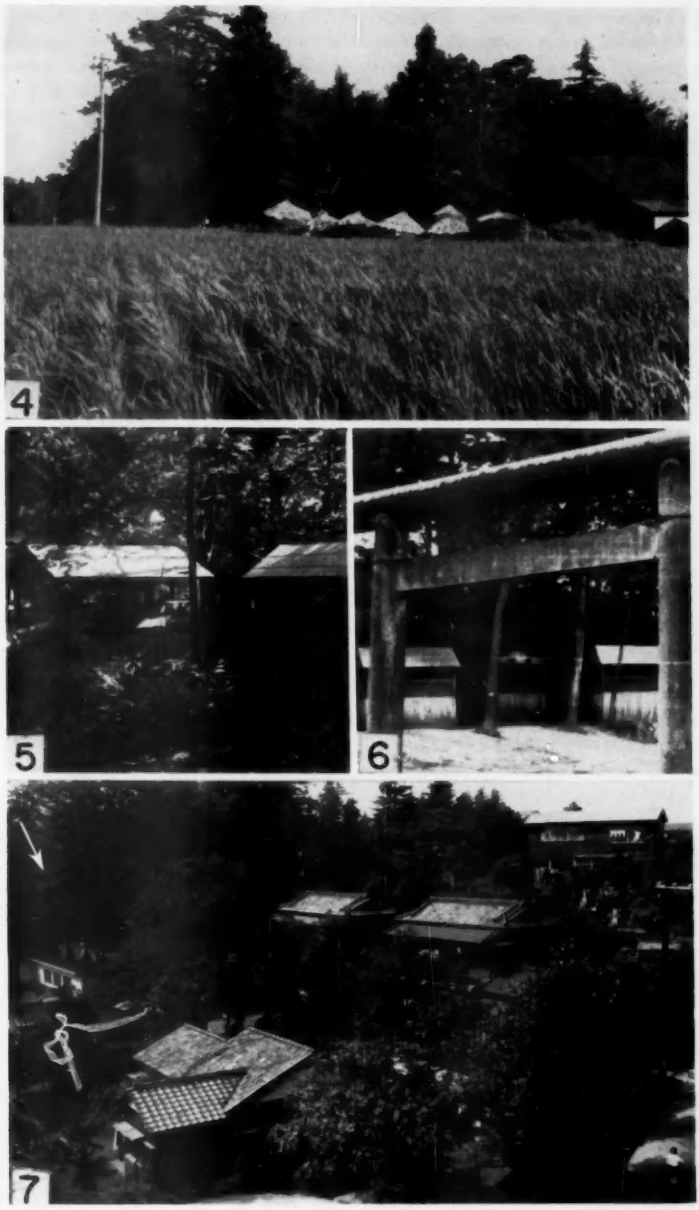
Designs of mosquito traps. Small ground traps were those employed for collecting virus infected mosquitoes.[3] Because large animals and man were used in 1956, larger traps[2, 11] (Figs. 1, 2, 5 and 6) were required. These modified Magoon traps were 72 x 96 x 78 inches and were covered by a peaked roof. The unfinished wooden bottom, screened midportion and upper wood and galvanized iron roof were built separately to facilitate transportation. Mosquito entranceways were similar to those described for ground traps[3] except that they were constructed of 18-mesh screen (a. in Fig. 2), and terminated in a horizontal opening 1 inch wide (b. in Fig. 2). The external vertical openings of the entranceways (c. in Figs. 1 and 2) were covered by mosquito-proof cloth mesh except during collection periods. Traps suspended from

* Current addresses: 1st Army Area Laboratory, US Army, New York (M. B. F); University of Maryland, College Park (J. S.)

† In these studies, tropism means "moving in a particular direction in response to a special external stimulus."



FIGS. 1–3. The modified Magoon traps for pigs, birds and man are shown in Figures 1 and 2, and altitude traps suspended from trees in Figure 3.



FIGS. 4–7. The Zama Magoon traps were kept at a shrine in a wooded area surrounded by rice paddies (Figs. 4 and 5). The Tokyo traps (Fig. 6) were at a shrine (arrow, Fig. 7) in a residential district.

trees (Fig. 3) were similar in shape, but smaller than standard ground traps.[3] Each altitude trap had an entranceway for mosquitoes along each side and one at the rear. The inside opening at the apex of the entranceway was horizontal and 0.75 inches wide. A horizontal partition of hardware cloth confined birds to lower portions of the traps.

Bait in mosquito traps. Black-crowned Night Herons (BCNH) and Little Egrets (LE) were collected as fledglings at Shinhama in May or June of 1953 and 1956. Chickens (Chick) were reared at the laboratory during spring 1953 and were used during the summer as birds up to 6 months of age. Juvenile or adult Blue Magpies (B Mag) from Irumagun, Tree Sparrows (T Spar) from Daiganji or Setagaya, and Grey Starlings (G Star) from Daiganji or Hama Park in Tokyo, were netted during spring 1953. Dusky Thrushes (D Thru) were netted during fall 1952 near Toyama on the west coast of Japan and kept in the 406 MGL aviary until placed in mosquito traps in spring 1953.

Since animal size and surface area were possible factors in attracting mosquitoes (subsequently shown to be important for *C. tarsalis* and birds[6]), sufficient animals were used to provide similar total body surface areas in each trap. Thus more small birds were used per trap than large birds, *i.e.*, BCNH and LE-2; B Mag and D Thru-3; G Star-3 or 4 at Shinhama and 2 or 3 at Minami-gyotoku; Chick-4 at Shinhama and 3 or 4 at Minami-gyotoku; and T. Spar-8. In 1956 two pigs 3 to 6 months of age, one man and six or 12 BCNH were employed as bait in Magoon traps. The altitude traps in 1956 contained two BCNH. The humans were adults of Caucasian, Negro and Oriental races at Sagiyama and Tokyo, and Caucasian at Zama. Each was given formalinized mouse brain vaccine in June or July 1956. They slept under mosquito nets and occasionally blankets when traps were open for collections; lights and smoking were not permitted. Birds and pigs in traps were constantly supplied with water and food. Pigs ate a dried, powdered meal, BCNH and LE ate loaches, and other birds ate grain, a dried commercial bird food, hamburger and raisins.

Collection and identification of mosquitoes. In 1953 mosquitoes were collected daily from bird-baited ground traps and identified as described.[3] In 1956 three Magoon traps were open from 8 p.m. to 7 a.m. Sunday, Tuesday and Thursday nights at Sagiyama and Zama and on Monday, Wednesday and Friday at Tokyo. Mosquitoes were collected from traps by aspiration within 5 hours of closure (Fig. 2). From altitude traps in 1956, collections were made thrice weekly although traps were open to mosquitoes at all times.

RESULTS

The experimental findings are presented under four headings: 1) a comparison of seven different bird genera as attractors of mosquitoes; 2) a comparison of Black-crowned Night Herons, pigs and man as attractors; 3) the effect of altitude upon mosquito recoveries from bird-baited traps; and 4) the influence of microhabitats and individual trap variables upon mosquito collections. While primary attention was directed towards the behavior of *C. tritaeniorhynchus*, other species were trapped as well. These are mentioned in the following descriptions, since in many cases their behavior differed from that of the vector, *C. tritaeniorhynchus*.

Mosquitoes attracted to birds of seven genera in traps at Shinhama and Minami-gyotoku in 1953. In comparing numbers of mosquitoes attracted to birds at Shinhama and Minami-gyotoku in 1953, common birds with both high and low incidences of JE virus neutralizing (N) antibody[9] were selected (high = Black-crowned Night Herons, Dusky Thrushes, Blue Magpies, Tree Sparrows and Grey Starlings; low = Little Egrets and chickens).

Only *Culex tritaeniorhynchus* and *C. pipiens* were found in bird-baited traps at Shinhama and Minami-gyotoku in 1953 (Table 1). Traps with BCNH captured significantly more *C. tritaeniorhynchus* than traps with the other six species; yet chicken-baited traps equalled or bettered BCNH-baited traps in attracting *C. pipiens*; control traps without birds attracted no mosquitoes. *C. tritaeniorhynchus* were found in smaller numbers than *C. pipiens* except for BCNH- and LE-baited traps at Shinhama. *C. tritaeniorhynchus* catches were similar in size at the two locations whereas *C. pipiens* were more prevalent at Minami-gyotoku than at Shinhama. *C. tritaeniorhynchus* were scarce, but *C. pipiens* were abundant in traps baited with Blue Magpies, Tree Sparrows, Dusky Thrushes and Grey Starlings. Although only total mosquito collections for the summer are given in Table 1, daily collections showed essentially similar patterns of bait tropism. In general, results from duplicate traps were

TABLE 1

*Mosquitoes collected from small ground traps baited with birds of seven genera at Shinhama and Minami-gyotoku, May–August, 1953**

Mosquito	Location	Nos. of mosquitoes collected from pairs of traps baited with							
		BCNH	LE	Chick	B Mag	T Spar	D Thru	G Star	Empty trap
C. tritaeniorhynchus	Shinhama	4,170	267	322	1	1	0	0	0
	Minami-gyotoku	1,268	408	146	20	29	15	7	0
C. pipiens	Shinhama	472	116	430	46	48	5	35	0
	Minami-gyotoku	8,712	5,376	11,679	2,719	1,952	2,640	4,254	0

* Essentially all mosquitoes were collected during May–August; very few entered traps in April or September.

similar. Differences related to individual traps or to their immediate environment were evident in only three pairs of traps at Minami-gyotoku; these occasions are described and discussed below. However, they did not affect qualitatively the results shown in Table 1.

Mosquitoes attracted to birds, pigs and man in traps at Sagiyama, Zama and Tokyo in 1956. Since variations in zootropism of *C. tritaeniorhynchus* occurred among different birds (Table 1) and had previously been reported between large and small animals,[12] it became apparent that the relative attractiveness of birds, large animals and man for *C. tritaeniorhynchus* had to be compared simultaneously. BCNH were chosen since among birds they attracted the most *C. tritaeniorhynchus* (Table 1). Pigs were selected because they were suspected (and subsequently shown[15]) to be important in the ecology of JE virus near Tokyo.

Pig-baited traps attracted the most *C. tritaeniorhynchus* at each of three locations (Table 2); BCNH-baited traps collected fewer *C. tritaeniorhynchus* but more than man-baited traps. While man and pigs probably did not destroy any mosquitoes in traps, BCNH may have, since they were not confined to the lower portion of Magoon traps as they were in ground traps. *Anopheles sinensis*, like *C. tritaeniorhynchus*, was much more frequent in pig-baited traps than in bird- or man-baited traps. In contrast, *C. pipiens* was more uniformly attracted to all three hosts, although it was collected most frequently from BCNH-baited traps.

That the larger numbers of *C. tritaeniorhynchus* attracted to pigs was a function of the pig and not a peculiarity of the trap itself or its location, is supported by three facts: 1) results were similar in two locations, Sagiyama and Zama, separated by 30 to 40 miles (Figs. 8 and 9); 2) trap location bias was essentially eliminated at each study site by placing the traps close together along equidistant radii of a circle (Figs. 1, 5 and 6); and 3) two changes of trap positions at Sagiyama on August 7th and 21st failed to influence the size of collection from pig-baited Magoon traps (Fig. 8). In changing positions of traps, they were rotated 180 degrees to prevent a trap being placed over the former site of another trap where residual

TABLE 2

Mosquitoes collected from Magoon ground traps baited with pigs, birds and man at Sagiyama, Zama and Tokyo, June–September 1956

Mosquitoes	Location	Nos. of moquitoes collected from traps baited with		
		Pig	BCNH	Man
C. tritaeniorhynchus	Sagiyama	120,381	5,791	278
	Zama	4,123	112	16
	Tokyo	234	41	11
A. sinensis	Sagiyama	14,732	49	244
	Zama	566	2	5
	Tokyo	0	0	0
C. pipiens	Sagiyama	174	192	46
	Zama	202	443	61
	Tokyo	91	631	108

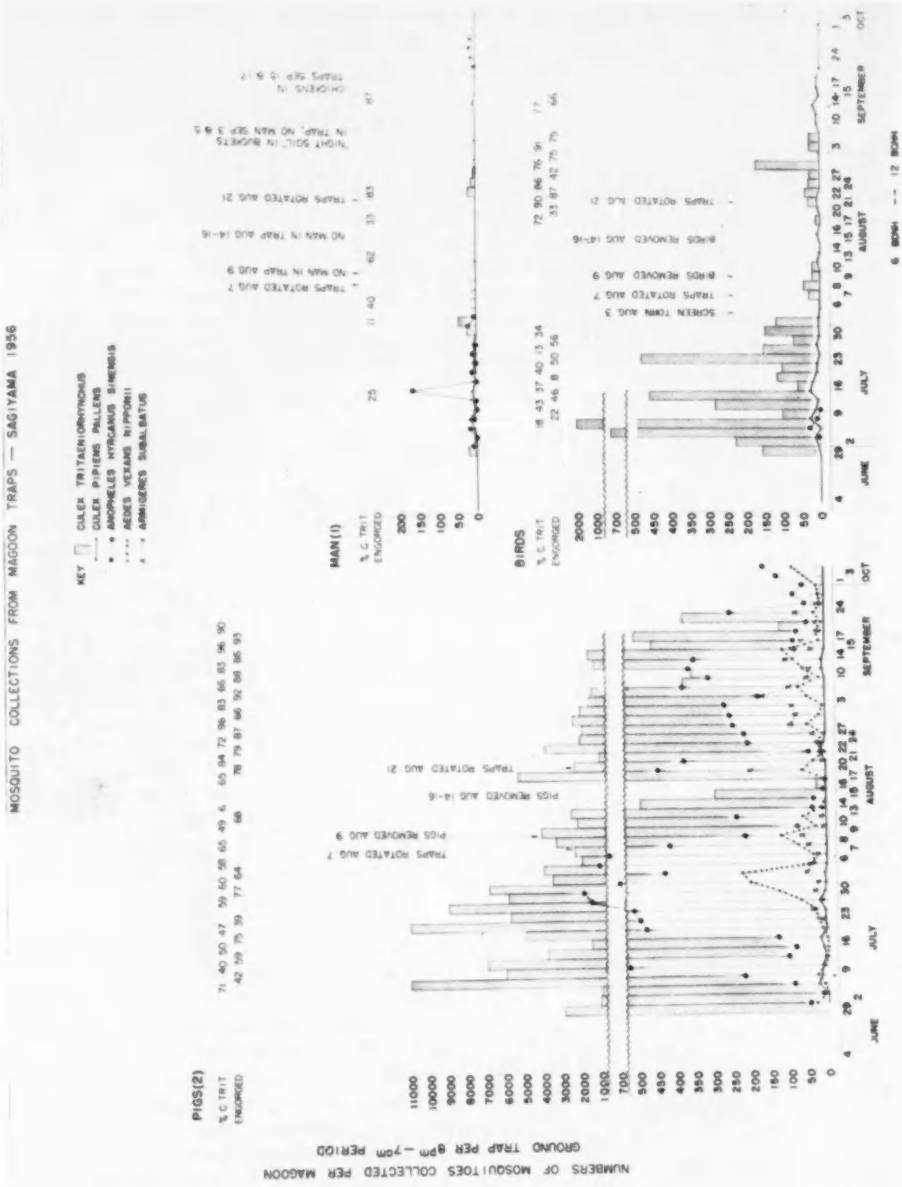

MOSQUITO COLLECTIONS FROM MAGOON TRAPS — SAGIYAMA 1956
KEY
CULEX TRITAENIORHYNCHUS
CULEX PIPIENS PALLENS
ANOPHELES HYRCANUS SINENSIS
AEDES VEXANS NIPPONII
ARMIGERES SUBALBATUS
PIGS(2)
% C TRIT
ENGORGED
MAN(1)
BIRDS
TRAPS ROTATED AUG 7
PIGS REMOVED AUG 9
PIGS REMOVED AUG 14-16
TRAPS ROTATED AUG 21
SCREEN TOWN AUG 3
BIRDS REMOVED AUG 9
BIRDS REMOVED AUG 14-16
NO MAN IN TRAP AUG 9
NO MAN IN TRAP AUG 14-16
CHICKENS IN TRAPS SEP 13 & 17
JUNE
JULY
AUGUST
SEPTEMBER
OCT
NUMBERS OF MOSQUITOES COLLECTED PER MAGOON GROUND TRAP PER 8PM — 7AM PER100

Fig. 8

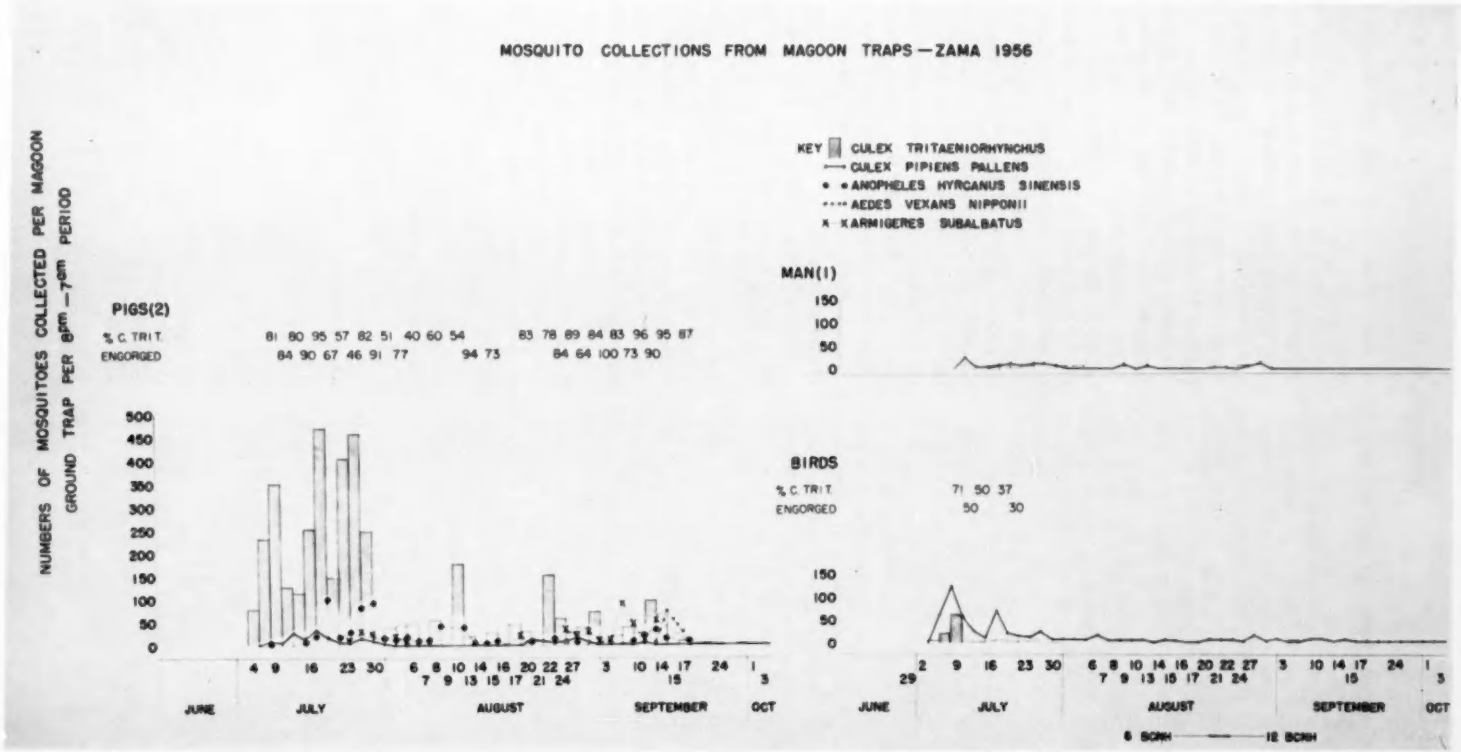

MOSQUITO COLLECTIONS FROM MAGOON TRAPS — ZAMA 1956
NUMBERS OF MOSQUITOES COLLECTED PER MAGOON GROUND TRAP PER 8cm — 7cm PERIOD
KEY
CULEX TRITAENIORHYNCHUS
CULEX PIPIENS PALLENS
ANOPHELES HYRCANUS SINENSIS
AEDES VEXANS NIPPONII
X ARMIGERES SUBALBATUS
PIGS(2)
% C. TRIT. ENGORGED
81 80 95 57 82 51 40 60 54 83 78 89 84 83 96 95 87
84 90 67 46 91 77 94 73 84 64 100 73 90
MAN(1)
BIRDS
% C. TRIT. ENGORGED
71 50 37
50 30
JUNE JULY AUGUST SEPTEMBER OCT
6 SCNH 12 SCNH

FIG. 9

factors such as odors from pigs and birds might attract mosquitoes.

Of five species of mosquitoes trapped in this experiment, *C. tritaeniorhynchus*, *A. sinensis*, *C. pipiens*, *Aedes vexans nipponii* and *Armigeres subalbatus*, only the first three were found in significant numbers (Table 2, Figs. 8 and 9). *C. tritaeniorhynchus* and *C. pipiens* were found at each location, but *A. sinensis* was caught only at Sagiyama and Zama.

Since transmission of JE virus requires that vector mosquitoes bite animals, it was important to evaluate engorgement of *C. tritaeniorhynchus* as well as numbers entering traps. Not all mosquitoes that enter traps engorge; in fact engorgement rates can be independent of attraction rates, *e.g.*, *C. tarsalis* and birds.[6] In addition, it was essential to learn whether *C. tritaeniorhynchus* entering traps actually bit the animal bait or whether they engorged upon other animals outside traps before entering. Since previous studies with precipitin technics had shown that blood in mosquitoes caught in animal-baited traps and resting stations usually originates from the species of animal used as bait,[1] no experiments of this sort were performed here. However, it is noteworthy that on three occasions (August 9, 14, 15 and 16) when pigs were removed from traps at Sagiyama, collections of engorged mosquitoes promptly decreased from 68 to 6, 0 and 0%, even though nonengorged *C. tritaeniorhynchus* continued to be trapped (Fig. 8). In fact, engorged mosquitoes were collected from an empty trap only once (August 14, Sagiyama pig trap); these were 30 engorged *C. tritaeniorhynchus* and three *A. sinensis* (1 to 2% of the previous day's collections) and may well have represented a few mosquitoes overlooked the day before. Thus, the high percentages (60 to 100%) of engorged *C. tritaeniorhynchus* in animal-baited traps (Figs. 8 and 9), absence of engorged but not of nonengorged mosquitoes in traps without bait (Fig. 8), and the fact that pigs and birds employed as bait in traps became infected by JE virus,[4, 15] strongly suggested that *C. tritaeniorhynchus* fed upon animals in traps.

Mosquitoes attracted to birds in altitude traps. Since birds at Sagiyama were infected in nests up to 60 feet above ground, the question arose whether *C. tritaeniorhynchus* or another arthropod was the vector at such heights. Thus, in 1956 a qualitative study was carried out with birds in six mosquito traps at 24 to 50 feet above ground to learn which mosquitoes were present in tree tops.

Small numbers of adult female *C. tritaeniorhynchus* were found (less than 20 per week) in each altitude trap consistently throughout July. and in areas 17, 19 and 21 (Fig. 4, Article I) during several weeks of August. No JE virus was recovered from 41 *C. tritaeniorhynchus* tested as four pools, although nine of 11 BCNH in altitude traps developed antibody to JE virus during August and September, establishing that avian infection did occur in mosquito traps at tree-top levels. *C. pipiens* and *C. bitaeniorhynchus* were found simultaneously (usually less than 20 and 10 per week, respectively). On two occasions *C. pipiens* reached 20 to 30 per week in area 17 and one week (9–16 July), 40 were caught in area 20. Other species were rarely encountered and then only as single individuals, *viz.*, *Aedes albopictus* (twice), and *Aedes vexans nipponii*, *Culex vorax*, *Culex orientalis* and *A. sinensis* each once, and all in areas 19, 20, or 22, thirty-three or more feet above ground.

Effect of trap location and trap proper upon mosquito collections and recovery of JE virus. On four occasions during these experiments, mosquito collections were biased by factors relating to trap location within a study site or to the trap itself. Since microhabitats and trap design were thought to be factors influencing entry of mosquitoes into animal-baited traps experiments were in general designed to avoid them as variables. For example, in 1953 at Shinhama, when different bird species were tested for their ability to attract *C. tritaeniorhynchus*, bird species were rotated among the different trap locations on several occasions during the summer. Also to minimize variations in attractiveness of individual birds for mosquitoes,[6] pairs rather than single birds were used in each trap. However, because of limited manpower in 1953, interchange of bird species among traps was not feasible at Minami-gyotoku where traps were in different yards separated by distances of 100 feet to 1 mile. As a result, three mosquito recoveries were influenced by trap location within a study site (microhabitat) (Table 3). Collections of *C. tritaeniorhynchus* from the BCNH-baited trap in area 1 were less than 10% of collections from the trap in area 2. Likewise differences occurred between *C. tritaeniorhynchus* collections from egret-baited traps in areas 3 and 4, and between *C. pipiens* from chicken-baited

TABLE 3

Biased mosquito collections related to locations of traps within study sites

Study site and year	Trap(s) in area(s)	Birds in traps	Nos. of mosquitoes collected during June–September	
			C. tritaeniorhynchus	*C. pipiens*
Minami-gyotoku, 1953	1	BCNH	71	2,374
	2	BCNH	1,172	3,548
	3	LE	20	1,585
	4	LE	384	1,773
	5	Chicken	107	7,458
	6	Chicken	43	2,445
Shinhama, 1956	1, 2	BCNH	836	64
	3, 4	BCNH	2,281	83
	5, 6	BCNH	542	462
	7, 8	BCNH	750	305
Sagiyama, 1956	9, 10	BCNH	3,596	271
	11, 12	BCNH	3,838	530
	13, 14	BCNH	13,783	529
	15, 16	BCNH	2,305	327

TABLE 4

Inherent trap bias in mosquito collections from BCNH-baited ground traps 30 feet apart at Sagiyama, 1956

Area	27 June–31 July		1 Aug.–1 Oct.	
	Trap no.	No. *C. trit.* collected	Trap no.	No. *C. trit.* collected
9	9	572	10	265
10	10	2708	9	49

traps in areas 5 and 6; collection differences were consistent during each week between June and September. It is noteworthy that whatever factor(s) caused differences between traps for one mosquito species did not necessarily affect the other simultaneously.

Microhabitats also affected mosquito yields from bird-baited traps in 1956. At Shinhama, two traps in areas 3 and 4 consistently yielded 2 to 4 times more *C. tritaeniorhynchus* than two traps in areas 5 and 6 (Table 3) despite the traps being separated by less than 200 feet (Fig. 3, article I). At Sagiyama, traps in areas 13 and 14 yielded 3 to 6 times more *C. tritaeniorhynchus* than other traps (Table 3, Fig. 10). These differences were not due to peculiarities among individual baits, since the birds were replaced several times during August as they became infected by JE virus. Unfortunately, the cause or causes of these differences in mosquito collections between trap locations is now impossible to determine in retrospect; apparently they were related to microhabitats, but whether they reflected mosquito breeding, flight activity, direction of air currents, or focal use of insecticides (only possible at Sagiyama) is unknown. Two ground traps in areas 15 and 16 also differed from the other six Sagiyama traps in that they failed to yield mosquitoes containing virus, although the same numbers of *C. tritaeniorhynchus* (5 to 25/day) were collected from them as from other traps (Fig. 10). Areas 15 and 16 were dark and in a dense cluster of tall bamboo (Fig. 4, Article I), and perhaps this difference in microhabitat contributed to the failure to capture virus-infected mosquitoes.

Equally hard to explain was the perplexing difference between *C. tritaeniorhynchus* recoveries from two BCNH-baited traps within 30 feet of each other at Sagiyama (areas 9 and 10, Fig. 4, Article I). During July the trap in area 10 captured 4 to 5 times as many *C. tritaeniorhynchus* as the trap in area 9 (Table 4). On August 1st the traps were interchanged, but the trap originally in area 10 continued to collect more mosquitoes even though it was now in area 9 (Table 4, Fig. 10). Although the trap originally in area 9 was made in 1953 and the trap in area 10 in 1956, their designs and component parts were similar and provided no readily apparent explanation for differences in mosquito yields. Variations in attractiveness of individual birds for *C. tritaeniorhynchus* did not explain the unusual results since collection differences were not influenced by changing individual birds. Thus, these observations apparently represent bias of results related to the trap *per se*.

Mosquitoes collected from traps recently emptied of animal bait. Baits were removed from traps twice at Sagiyama in 1956 to learn whether *C. tritaeniorhynchus* would continue to enter traps recently emptied of bait. On the first occasion (August 9th), essentially the same numbers of *C. tritaeniorhynchus* entered as on August 8th and 10th. However, when the animals were removed for 3 consecutive days, August 14th–16th, collections progressively decreased to less than 50 (chiefly unengorged) mosquitoes from the pig trap and essentially zero from the bird- and man-baited traps (Fig. 8). These observations sug-

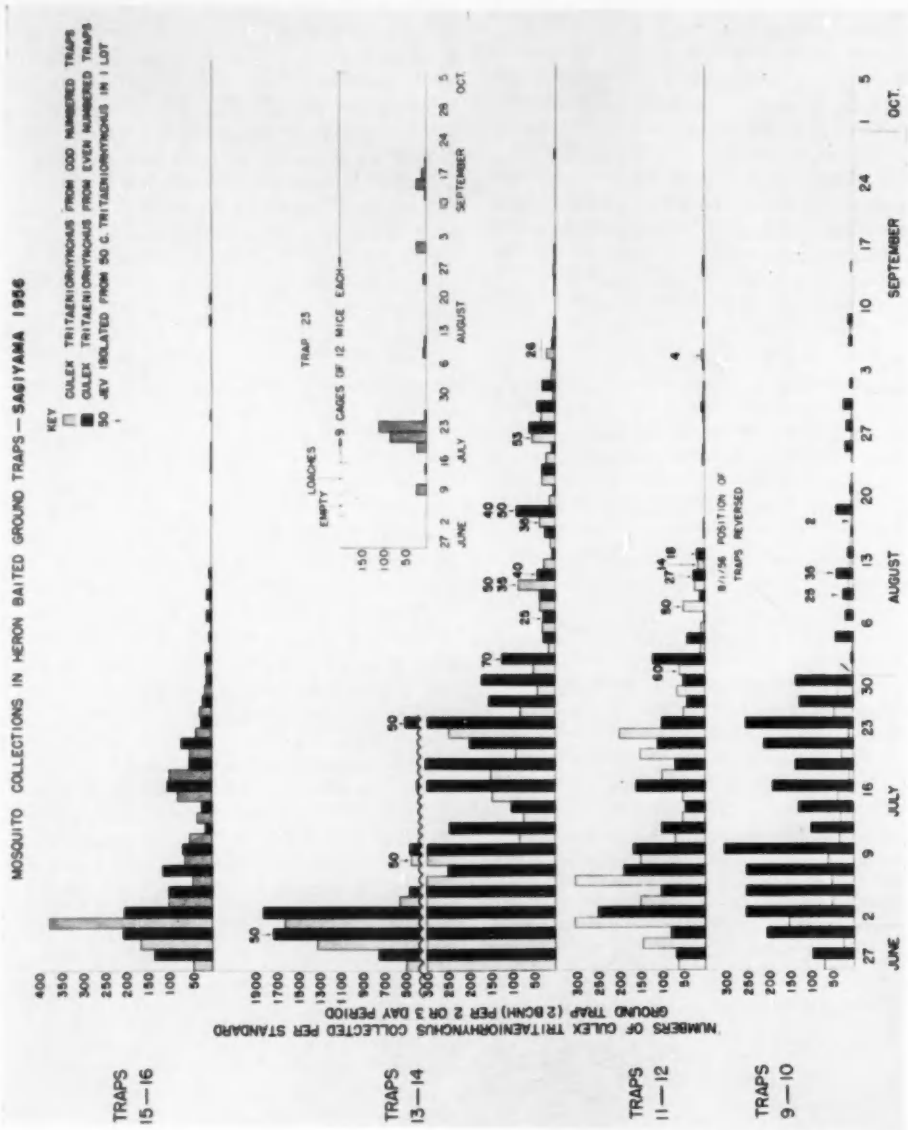

MOSQUITO COLLECTIONS IN HERON BAITED GROUND TRAPS — SAGIYAMA 1956
KEY
CULEX TRITAENIORHYNCHUS FROM ODD NUMBERED TRAPS
CULEX TRITAENIORHYNCHUS FROM EVEN NUMBERED TRAPS
JEV ISOLATED FROM 50 C. TRITAENIORHYNCHUS IN 1 LOT
LOADED
EMPTY
TRAP 23
9 CAGES OF 12 MICE EACH
POSITION OF TRAPS REVERSED
NUMBERS OF CULEX TRITAENIORHYNCHUS COLLECTED PER STANDARD GROUND TRAP (2 BCNH) PER 2 OR 3 DAY PERIOD
TRAPS 15—16
TRAPS 13—14
TRAPS 11—12
TRAPS 9—10
JUNE
JULY
AUGUST
SEPTEMBER
OCT.

FIG. 10

gested that whatever attracted *C. tritaeniorhynchus* into traps, slowly, rather than promptly, disappeared after removal of bait such as pigs.

DISCUSSION

The present study established that the zootropism of *C. tritaeniorhynchus* was similar in three areas near Tokyo. Proximity of this mosquito to large populations of one animal host did not affect its zootropism. For example, *C. tritaeniorhynchus* reared near ardeids at Sagiyama were not so conditioned to biting ardeids that they failed to be attracted to pigs or to man; in fact, they behaved exactly like those from another rural area without ardeids. Furthermore *C. tritaeniorhynchus* close to humans in Tokyo did not fly toward man in traps more frequently than toward pigs or birds. The smaller collections of *C. tritaeniorhynchus* from urban regions (Minami-gyotoku and Tokyo) than from rural areas suggests that this mosquito prefers areas minimally inhabited by man, and overflows into human habitations only under pressure, for example, of an increasing mosquito population. Such a rural distribution of *C. tritaeniorhynchus* would tend to keep infected mosquitoes from contact with man and limit the spread of human epidemics of JE.

However, not all species of mosquitoes behaved uniformly at different geographic sites. *C. pipiens* collections at Shinhama in 1953 differed by 10 to 94 fold among traps baited with different birds (Table 1) and only small numbers were caught when certain birds were employed as bait. Contrariwise, large numbers were found in traps at Minami-gyotoku regardless of the bird species employed as bait, and mosquito catches varied less than 6-fold among the different birds used as bait. Thus, *C. pipiens* at Shinhama in 1953 seemed to be more selective in its zootropism towards birds of different species than at Minami-gyotoku.

Trap location in the Kanto Plain failed to affect the predominance of *C. tritaeniorhynchus* attracted into pig-baited traps. These findings agree with those of Sasa and Sabin[12] who found in one test at Okayama, Japan that pigs attracted more *C. tritaeniorhynchus* than other species. On the other hand, the geographic location of traps affected the relative proportions of mosquitoes attracted to man and birds. At Sagiyama man-baited traps yielded chiefly *C. tritaeniorhynchus* and *A. sinensis* but at Zama and Tokyo *C. pipiens* was predominant, as observed by Kitaoka in 1948 at man-baited resting stations in Tokyo.[10] Inconsistent collections of mosquitoes attracted to bird-baited traps have also been recorded by others. For example, chicken-baited traps at Okayama, Japan, in 1946, collected approximately equal numbers of *C. pipiens* and *C. tritaeniorhynchus*,[12] yet in Tokyo, Sasa found that they captured more *C. pipiens* than *C. tritaeniorhynchus* in 1948 and vice versa in 1949.[13] In this study BCNH at Sagiyama attracted mostly *C. tritaeniorhynchus* whereas in Tokyo they attracted mostly *C. pipiens*. The reason(s) for discrepancies in mosquito collections among animal baits at different locations is unknown. Whether they reflect qualitative or quantitative differences in mosquito populations is uncertain, but the data suggest that behavior of *C. pipiens* differs by geographic location. Fortunately *C. pipiens* is not an important natural vector of JE virus so that behavioral differences among geographically separated populations play little if any role in virus ecology near Tokyo.

The altitude trap experiments provided circumstantial evidence that *C. tritaeniorhynchus* is the vector of JE virus among birds in tree tops at Sagiyama. *C. tritaeniorhynchus* was found in trees as high as 43 to 50 feet above ground, and BCNH in traps became infected by JE virus. Although these data do not prove unequivocally that *C. tritaeniorhynchus* carries virus among birds in tree tops at Sagiyama, it seems unlikely that other mosquitoes collected in trees, *viz. C. pipiens* and *C. bitaeniorhynchus*, could be important vectors since they were rarely if ever infected at ground level.[3]

During analysis of these experiments, it became evident that the results of mosquito tropism studies must be compatible with other knowledge of virus ecology. For example, if merely three observations are put together, 1) that *C. pipiens* is a frequent bird-biting mosquito (Tables 1 and 2),[12] 2) that birds can serve as sources of JE virus for mosquitoes;[4] and 3) that *C. pipiens* can transmit JE virus in the laboratory,[8] the conclusion could be reached that *C. pipiens* is probably the most frequently infected mosquito and the vector of JE virus among birds at heronries. Obviously such an extrapolation is invalid since *C. pipiens* was almost never infected by JE virus[3] at Shinhama and Sagiyama despite its predilection to enter bird-baited traps and bite birds. Although the low infection rate of *C. pipiens* remains unexplained, one possibility is that it requires for in-

fection, ingestion of JE virus at higher blood levels than are present in ardeids[5, 7] whereas *C. tritaeniorhynchus* becomes infected by ingestion of small quantities of virus.[7] Regardless of the explanation, however, the fact is apparent that extrapolations from one facet of JE virus ecology to another are potentially incorrect unless they are checked by field experiments.

In conclusion, four positive relationships between *C. tritaeniorhynchus* behavior and JE virus ecology at Shinhama and Sagiyama can be derived from these studies. 1) The marked tendency of *C. tritaeniorhynchus* to bite pigs in traps helped to explain the high frequency of virus infection in shoats near Sagiyama during August 1956[15] and suggested that pigs may be highly efficient in seeding the vector mosquito population with virus. 2) The fact that *C. tritaeniorhynchus* bit BCNH more frequently than egrets accounted at least in part, for the higher incidence of BCNH infection than of egret infection at the heronries.[4] 3) The flights of *C. tritaeniorhynchus* to altitudes of 43 to 50 feet at Sagiyama helped to explain spread of JE virus among birds that nest in tree tops. 4) The influence of trap location on *C. tritaeniorhynchus* collections indicated that microhabitats can influence the ecology of virus.

SUMMARY

At the Shinhama heronry and nearby Minamigyotoku village near Tokyo, Japan, in 1953, *C. tritaeniorhynchus*, the major vector of Japanese encephalitis virus in Japan, entered traps baited with Black-crowned Night Herons in numbers 3 to 15 times greater than traps containing egrets or chickens; Blue Magpies, Tree Sparrows, Dusky Thrushes, and Grey Starlings failed to attract significant numbers. In 1956, at two rural and one urban location near Tokyo, pigs attracted and were bitten by many more *C. tritaeniorhynchus* than BCNH or man. *C. tritaeniorhynchus* were found in traps as high as 50 feet above ground at the Sagiyama heronry. The behavior of the vector mosquito explained in part the different incidences of virus infection among bird species, the high incidence of swine infection in nature, and infection of nestling birds high in trees at Sagiyama. Whatever animal mechanism exists for attracting *C. tritaeniorhynchus*, it persisted in traps for 1 to 3 days after removal of the animal.

Other mosquitoes behaved differently from *C. tritaeniorhynchus*. *C. pipiens* frequently entered traps with all seven species of birds and was attracted to BCNH in traps more than to pigs. *A. sinensis* was found in significant numbers only in pig-baited traps in rural areas. Other species were recovered only in small numbers.

Microhabitats affected entry of mosquitoes into traps and necessitated study of multiple trap sites with duplicate traps. On one occasion *C. tritaeniorhynchus* collections were influenced by factors related to the individual trap *per se* and independent of trap design or location. These studies emphasized that predictions of virus ecology from knowledge of mosquito behavior alone may be erroneous.

REFERENCES

1. Barnett, H. C., 1950. Annual Professional Report, 406th Medical General Laboratory, pp. 186–191.
2. Bates, M., 1944. Notes on the construction and use of stable traps for mosquito studies. *J. Nat. Malaria Soc.*, **3:** 135–145.
3. Buescher, E. L., Scherer, W. F., Rosenberg, M., Gresser, I., Hardy, J. L., and Bullock, H. R., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. II. Mosquito infection. *Am. J. Trop. Med. & Hyg.*, **8:** 651–664.
4. Buescher, E. L., Scherer, W. F., McClure, H. E., Moyer, J. T., Rosenberg, M., Yoshii, M. and Okada, Y., 1959b. Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. *Am. J. Trop. Med. & Hyg.*, **8:** 678–688.
5. Buescher, E. L., Scherer, W. F., and Rosenberg, M., 1959c. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses of birds. *J. Immunol.*, **83:** 605–613.
6. Dow, R. P., Reeves, W. C., and Bellamy, R. E., 1957. Field tests of avian host preference of *Culex tarsalis* COQ. *Am. J. Trop. Med. & Hyg.*, **6:** 294–303.
7. Gresser, I., Hardy, J. L., and Scherer, W. F., 1958. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles, from infected pigs and chicks to susceptible pigs and birds. *Am. J. Trop. Med. & Hyg.*, **7:** 365–373.
8. Hammon, W. McD., Rees, D. M., Casals, J., and Meiklejohn, G., 1949. Experimental transmission of Japanese B encephalitis virus by *Culex tritaeniorhynchus* and *Culex pipiens* var. *pallens*, suspected natural vectors. *Am. J. Hyg.*, **50:** 46–50.
9. Hammon, W. McD., Sather, G. E., and McClure, H. E., 1958. Serologic survey of Japanese B encephalitis virus infection in birds in Japan. *Am. J. Hyg.*, **67:** 118–133.
10. Kitaoka, M., Miura, T., and Ogata, T., 1950. Seasonal occurrence of mosquitoes in nature in Tokyo 1948 and 1949. Zoophilia of each species of mosquito. *Jap. Med. J.*, **3:** 331–347.

11. MAGOON, E. H., 1935. A portable stable trap for capturing mosquitoes. *Bull. Entomol. Res.*, **26:** 363–369.
12. SASA, M., AND SABIN, A. B., 1950. Ecological studies on the mosquitoes of Okayama in relation to the epidemiology of Japanese B encephalitis. *Am. J. Hyg.*, **51:** 21–35.
13. SASA, M., KANO, R., HAYASHI, S., KIMURA, M., MIURA, A., OYAMA, K., SATO, K., HOSOYA, H., AND HASEGAWA, K., 1950. Two years' observation on the seasonal activities and zoophilism of mosquitoes in Tokyo, by animal trap method. *Jap. J. Exp. Med.*, **20:** 509–517.
14. SCHERER, W. F., AND BUESCHER, E. L., 1959. Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. *Am. J. Trop. Med. & Hyg.*, **8:** 644–650.
15. SCHERER, W. F., MOYER, J. T., IZUMI, T., GRESSER, I., AND MCCOWN, J. M., 1959. Ecologic studies of Japanese encephalitis virus in Japan. VI. Swine infection. *Am. J. Trop. Med. & Hyg.*, **8:** 698–706.
16. THEILER, M., 1958. *Viral and Rickettsial Diseases of Man*, Philadelphia, J. B. Lippincott Co. Edited by Rivers and Horsfall, 3rd edition, chap. 15.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN

IV. Avian Infection*

E. L. BUESCHER, W. F. SCHERER, H. E. McCLURE,† J. T. MOYER,† M. Z. ROSENBERG, M. YOSHII AND Y. OKADA

Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

INTRODUCTION

The role of wild birds in the natural dissemination of Japanese encephalitis (JE) virus was uncertain in 1952 when the present ecologic study was begun. In 1937 Japanese investigators working in Okayama, Japan, recovered JE virus from two of 25 pools of blood obtained from 126 sparrows ("suzume"; species unknown) collected between 21 and 27 August.[11] Each isolate was established from a single diseased mouse of 7 to 15 inoculated with bird blood. No attempts are recorded to reisolate these strains, or to find antibody to JE virus in sera of wild birds. Subsequent attempts to study birds for natural infection with JE virus were reported by Kitaoka and his associates[10] who recovered a single strain of JE virus in 104 attempts from birds of at least 12 species confined in cages at the Ueno Zoo in Tokyo during the summers of 1946–1947. While the authors themselves questioned the validity of this virus isolation from a bamboo pheasant (*Bambusicola thoracica*), they did demonstrate neutralizing (N) antibody to JE virus in 13 of 205 individual birds of at least 13 species (all but one species were *Gallinaceae*) confined in zoos and on farms near Tokyo during the summers of 1946–1948. From the data obtained in 1937, 1946 and 1947, Mitamura and Kitaoka[12] concluded that wild birds were naturally infected with JE virus, but did not evaluate fully their role in the epidemiology of the disease. Smorodintzeff[17] also stated that birds were infected naturally in Eastern Soviet Siberia although details of the investigation were not presented. On the other hand, Sabin *et al.*,[13] and Bawell *et al.*,[1] upon finding no N antibody to JE virus in 62 chickens of Tokyo and Okayama prefectures during the spring and fall of 1946, concluded that this domestic fowl was infrequently infected in nature. In 1950–51 still another serologic survey of Japanese wild birds indicated that avian infection by JE virus was widespread.[7] Approximately 30% of sera contained N antibody, although the incidence varied greatly among species. For example, of three species nesting at the Shinhama heronry near Tokyo, (Black-crowned Night Herons, *Nycticorax nycticorax*, (BCNH), Little Egrets, *Egretta garzetta*, (LE), and Plumed Egrets, *Egretta intermedia*, (PE)), only BCNH and PE possessed antibody in 1950–51 (43% vs 0% vs 22%, respectively).

In 1952 an effort was therefore made to extend and evaluate these earlier observations by attempting to recover JE virus from wild birds near Tokyo. Initial baseline experiments perfomed in the laboratory showed that susceptible young birds developed viremia after subcutaneous inoculation of JE virus.[2, 7] Thus it was reasoned that if natural avian infection was similar to that induced experimentally, and if wild birds were as frequently infected as suggested by serologic survey, it should be possible to recover JE virus from them. This deduction proved correct since naturally occurring viremia was found in wild birds during the summer of 1952. The study was then extended during 1953–56 to determine the incidences and times of infection of birds in different areas, and to evaluate the role of avian infection in the ecology of JE.

MATERIALS AND METHODS

Study sites. Birds at 3 locations near Tokyo (Shinhama, Sagiyama[14] and Irumagun) and 2 rookeries near Nagoya (Tado and Unoyama) were tested for viremia and antibodies to JE virus. The Irumagun study site, approximately 10 miles northwest of Tokyo, (Fig. 2, article I), is similar in basic ecology to the areas surrounding

* Presented in part before the 55th General Meeting, Society of American Bacteriologists, New York, May, 1955.

† Present addresses: United States Army Medical Research Unit, Kuala Lumpur, Malaya (H. E. McC.); Center for Japanese Studies, University of Michigan (J. T. M.).

Sagiyama. It is an upland (in contrast to lowland or paddy) rural area devoted to cultivation of rice, barley, millet and garden vegetables, interspersed by large areas of mixed woodlands, and containing a number of widely scattered hamlets. Farmhouses line both sides of roads and lanes, and generally are sheltered by deciduous and coniferous trees, in which magpies commonly nest. The area supports a human population of approximately 1,200 per square mile. Each study site at Unoyama and Tado, near Nagoya, contains a large breeding colony of ardeids, and is similar in basic ecology to Shinhama and Sagiyama, respectively. Unoyama is 20 miles west of Nagoya proper, on a peninsula surrounded on three sides by ocean; the area is sparsely populated by man. Tado is situated in second-growth pine forest of a hillside overlooking a small valley devoted to paddy and dry land farming, and densely populated by man.

Birds. The predominant bird species at Shinhama, Sagiyama and Tado (BCNH, LE and PE) (Figs. 1–6) were studied more extensively than the less common species (Cattle Egrets, *Bubulcus ibis*, (CE) and Great Egrets, *Egretta alba*, (GE)). BCNH and Cormorants, *Phalacrocorax carbo* were tested at Unoyama and Blue Magpies, *Cyanopica cyaneus*, at Irumagun. The areas studied, the frequency of sampling bird populations, and the time intervals for viremia and antibody tests are indicated in Table 1. Studies of birds were terminated in September each year, because by that time most birds were flying and could not be captured.

Birds were captured and bled by teams of two to eight persons. At Shinhama, nestlings and flightless juveniles were readily caught by hand. At other study sites, where nesting was primarily in treetops, young were collected either directly from nests or as flightless juveniles from the ground after being dislodged from their perches. BCNH are readily distinguished by their plumage (Figs. 2, 3). However, nestling PE and LE (Figs. 4–6) are quite similar during the first 10 days of life. In 1952, the young of these two species were not distinguished; in succeeding years, nestling PE were differentiated from LE by skin and eye coloring and minor differences in plumage. Juveniles and adults were more readily identified; BCNH juveniles are brown (Fig. 2) and PE and LE, white (Figs. 4 and 5); BCNH adults are distinctive (Fig. 1), adult LE have yellow feet, and adult PE have black feet.

Captured birds ranged to 70 days of age, but most were between 1 and 30 days old. During 1952–55 the age of birds was estimated from overall body size, and was accurate to ±3–5 days. In 1956 greater accuracy of age estimates (±1–3 days) was obtained from measurements of third-primary wing feathers, the growth of which had been correlated with age.[9] Both methods for aging birds were more accurate during the first 21 days of life than in older birds.

After species and age were determined, each bird was banded on the leg or tagged on the wing and bled of 1 to 5 ml of blood from the external jugular vein into syringes previously wetted with 0.1 ml of heparin, 10 mg/ml.[2] After bleeding, birds were returned to their nests.

Ardeid population measurements. At Shinhama in 1956, nestlings were counted by two trained ornithologists from late June, until nestlings disappeared in September. Once each week these observers located essentially all nests in the refuge (Fig. 3, article 1) and counted nestlings. BCNH, LE and PE were classified as 1, 2, 3, or more than 3, weeks of age (fledglings) on the basis of body size and plumage development.

Isolation of virus from birds. Birds over 6 days of age were selected for virus isolation attempts to reduce the chance of maternal antibody preventing viremia.[3] Whether this decision actually influenced recovery of virus is discussed elsewhere.[15] Immediately upon withdrawal, whole blood was inoculated intracranially in 0.03-ml volume into 6 to 10 weanling mice which had been carried to the study site. In 1956 approximately one-half of the ardeid and all magpie bloods were also inoculated into 1 to 4-day-old mice from one litter (0.01 intracerebrally and 0.03 ml subcutaneously). Mice were identified by bird number, and observed at the laboratory for 14 to 21 days. Brains of mice showing signs of central-nervous-system disease were either prepared into complement-fixing (CF) antigens for test to identify the virus as JE,[4] or were passaged again to mice from which CF antigens were subsequently prepared. No blind passages of brain suspensions from normal-appearing mice were made.

Antibody tests. Blood was refrigerated and plasma removed at the laboratory within 1 to 3 days of collection in the field; plasma was stored at −20°C. Neutralization tests were performed on unheated plasma with M1/311 strain of JE virus as described elsewhere.[5] Log neutralization indices (LNI) of 0 to 0.9 were considered negative,

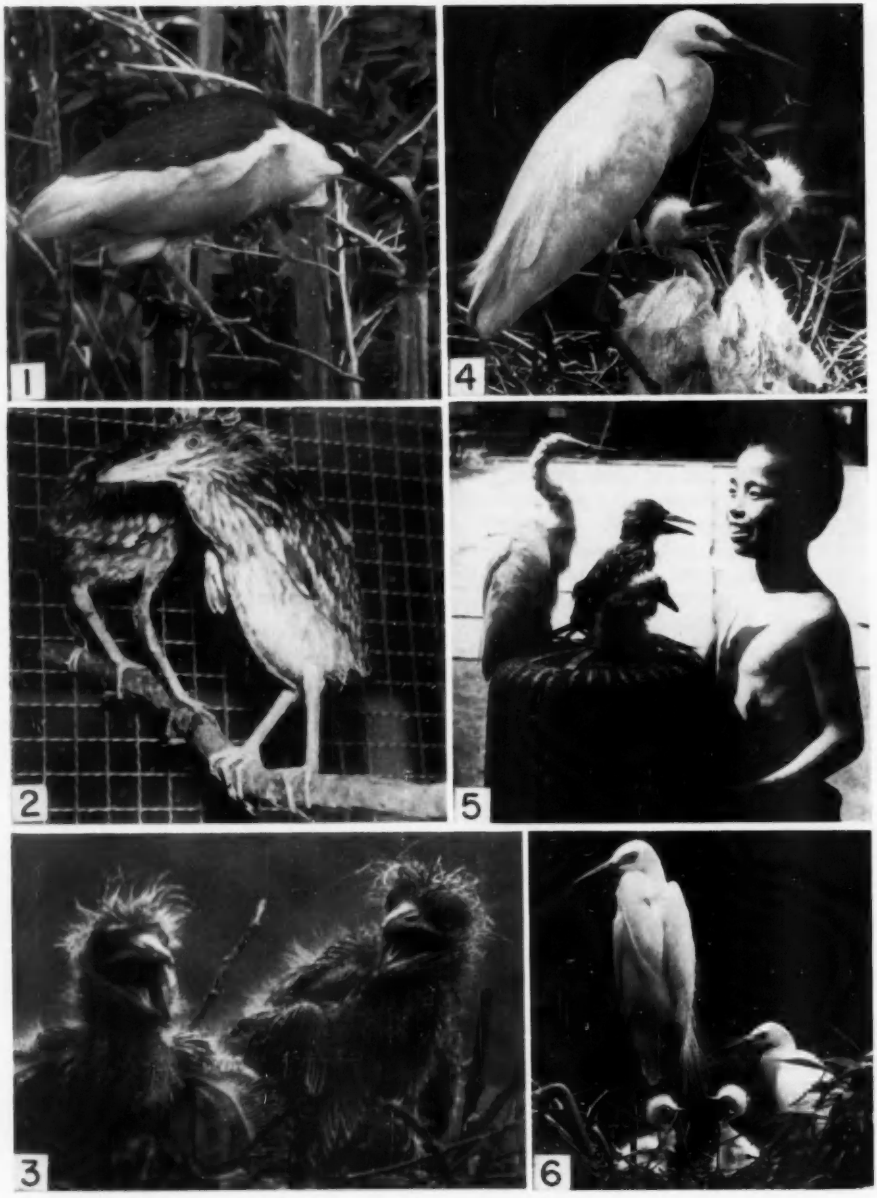


FIG. 1. Adult Black-crowned Night Heron (*Nycticorax nycticorax*).
FIG. 2. Juvenile Black-crowned Night Herons, in aviary.
FIG. 3. Black-crowned Night Heron nestlings, age approximately 10 days.
FIG. 4. Adult and young Little Egrets (*Egretta garzetta*), age approximately 15 days.
FIG. 5. Juvenile Plumed Egret (*Egretta intermedia*), and Black-crowned Night Herons.
FIG. 6. Plumed Egrets and young, age approximately 5 days.
Figs. 1, 3, 4, 5, and 6 Photos, Tokutaro Tanaka, Kitaurawa, Japan. Fig. 2 Photo, U. S. Army.

TABLE 1

Times and frequencies of testing free-living birds for infection with JE virus

Year	Study area	Sampling frequency/ week	Viremia tests		Antibody tests	
			Birds tested/week	Study interval	Birds tested/week	Study interval
1952	Shinhama	1	9–22	May 12–Sept 4	(total = 65)	Sept–Dec
1953	Shinhama	2	15–44	June 7–Aug 30	n.d.*	
	Irumagun	2–4	18–31	July 26 Aug 16	n.d.	
1954	Shinhama	1	21–52	July 26–Sept 13	50–75	May 18–Sept 13
	Sagiyama	1	37–53	July 26–Sept 13	50–75	May 18–Sept 13
	Irumagun	2–4	(total = 69)	July–Aug	(total = 59)	July–Aug
1955	Shinhama	1	3–51	July 22–Sept 8	25–50	July–Sept 8
	Sagiyama	1	9–50	July 18–Sept 20	25–50	May 15–Sept 20
	Irumagun	2–4	(total < 10)	July–Aug	(total = 46)	June–Aug
	Tado	2 or 3	n.d.		20–40	June–Aug
	Unoyama	2 or 3	n.d.		2–10	Mar–Aug
1956	Shinhama	1	10–55	July 18–Sept 5	50–100	May–Sept 5
	Sagiyama	1	3–36	June 26–Sept 4	40–60	May–Sept 4
	Irumagun	1	(total = 60)	July–Aug	(total = 237)	June–Aug

* n.d. = not done

1.0 to 1.7, equivocal, and greater than 1.7 positive. Hemagglutination-inhibition (HI) tests were performed on serially diluted, heated (56°C for 30 min.), acetone-extracted plasmas.[5] Inhibition at the lowest initial plasma dilution (1:10) or higher, was considered positive. All plasmas from the same bird were usually tested simultaneously.

RESULTS

Attempts were made to recover JE virus from wild birds to show unequivocally that avian infection occurred, to obtain crude estimates of time and incidence of infection, and to validate interpretation of antibody tests as measures of recent infection with JE virus. In studying wild birds, approximately 5% of the total avian populations, or up to 10% of nestlings at Shinhama and Sagiyuma were tested each year, *i.e.*, 200 to 300 birds studied/5000 total ardeids or up to 2000 nestlings (see *Discussion* and Fig. 7).

As the study progressed, it became apparent that tests for HI antibody reflected time and incidence of natural avian infection more accurately than virus isolation studies, because HI antibody, which appears uniformly after infection,[2] persists longer than viremia and the test is simpler to perform on large numbers of specimens than are virus isolations. Since maternal HI antibody is rare in herons over 15 days of age,[3] and since no other B Group virus exists in the studied areas near Tokyo,[14] HI titers of 1:10 or greater in older birds could be interpreted as evidence of recent infection with JE virus.

JE virus from birds at Shinhama and Sagiyama. JE virus was recovered from birds each year during 1952–1956 (Table 2). Fifty-four of 68 strains were from free-living birds in the study areas; 14 strains were from sentinel BCNH in mosquito traps. Of the 54 strains from free living birds, 35 were from wild BCNH, 16 from PE and three from LE. Of all 68 virus strains, ten were identified by CF tests of antigens made from brains of initially-inoculated mice (*i.e.*, first passage) and 58 from brains of second-passage mice. Virus strains recovered from two of three naturally infected birds in 1953 (1 free-living PE and 1 sentinel BCNH) were reisolated from frozen aliquots of plasma 2 months after initial isolation from blood and, in 1956, virus was recovered twice from the same bird, from bloods obtained on 12 and 19 August (8 and 15 days of age) (Table 2).

In 1956 approximately one-half of the bird bloods from Shinhama and Sagiyama were inoculated simultaneously into 3 to 4-week-old and 1 to 4-day-old mice in the field to determine whether a) the suckling mouse was a more sensitive indicator for unpassaged JE virus from birds than the weanling, and b) whether viruses other than JE might be recovered in suckling mice. No virus other than JE virus was isolated from bird

TABLE 2

JE virus from Black-crowned Night Herons, Plumed and Little Egrets, and Blue Magpies, 1952–1956

Study site	Year	Free-living birds						BCNH in mosquito traps	
		Isolation attempts*	Virus strains					Isolation attempts*	Virus strains
			BCNH	PE	LE	B Mag	Total		
Shinhama	1952	304	5	0	0	—	5	52	1
	1953	330	0	1	0	—	1	28	2
	1954	314	2	1	0	—	3	—	
	1955	289	16†	1	1	—	18	—	
	1956	257	7	1	2	—	10	107	7
Sagiyama	1954	348	1	3	0	—	4	—	
	1955	329	1	8	0	—	9	—	
	1956	238	3	1	—	—	4	240	4
Irumagun	1953	101	—	—	—	0	0	—	
	1954	90	—	—	—	0	0	—	
	1956	60	—	—	—	0	0	—	
Totals		2620	35	16	3	0	54	427	14

* Virus isolations were attempted from free-living birds 6 to 70 days of age, and from sentinel BCNH 1 to 4 months of age, except in 1953 when birds in traps were 9 months or older. Repeat bleedings of free-living birds occurred by consecutive years in the following frequencies at Shinhama; .05–.10, .15, .26 (combined with Sagiyama) .13, and .04; at Sagiyama, .38 (1955) and .09; at Irumagun, .66, .22 and 0. Since BCNH in traps were bled serially, the frequencies of repeat bleeding were greater, and were, chronologically, at Shinhama .92, .86 and .70; at Sagiyama, .70.

† JE virus was recovered twice, 7 days apart, from 1 BCNH.

bloods. Eleven strains of JE virus were recovered in both ages of mice, three strains only in suckling mice and one strain only in weanling mice. In retrospect, it was impossible to be certain that the blood yielding virus only in weanling mice had actually been inoculated into the suckling mice so labelled, and a technical error of omission may have occurred to account for this finding. Since only three of 15 strains were recovered solely in suckling mice, the difference in sensitivity to infection with unpassaged JE virus from birds between suckling and weanling mice did not seem to justify the added expense and problems attendant on the use of sucklings. Their continued use in search of other viruses is justified however.

TABLE 3

*Times of JE virus isolations from free-living and sentinel herons and egrets, 1952–1956**

Study site	Year	Dates of first and last virus isolations	
		Free-living birds	Sentinel BCNH
Shinhama	1952	7–21 Aug	21 Aug
	1953	2 Aug	11–16 Aug
	1954	19–23 Aug	not tested
	1955	29 July–30 Aug	not tested
	1956	8–28 Aug	22 Aug–5 Sept
Sagiyama	1954	30 Aug–6 Sept	not tested
	1955	1–23 Aug	not tested
	1956	25 July–28 Aug	14–21 Aug

* For both free-living and sentinel birds, testing was begun at least a week before the initial virus isolation, and continued into early September when essentially all nestlings had fledged, and the supply of susceptible sentinel birds was exhausted.

Time of avian infection at Shinhama and Sagiyama. Recovery of JE virus from wild birds was confined to a limited period in late summer each year (Table 3). Initial isolations of virus were obtained at approximately the same time each summer at Shinhama (29 July–19 August), and viremic birds were detected for 2 to 4 weeks thereafter. Similar intervals for avian infection were observed at Sagiyama in 1955 and 1956, although the time of appearance of virus in birds in 1954 was later than at Shinhama (Table 3). In 1952, 1953 and 1956 at Shinhama, and 1956 at Sagiyama, virus was recovered from free-living birds 1 to 3 weeks before it was first isolated from sentinel BCNH in mosquito traps, probably because greater numbers of free-living than sentinel birds were tested each week for virus.

In 1956, avian infection was also timed by weekly HI antibody tests of sentinel birds in mosquito traps. To estimate time of infection, 7 days are subtracted from the date HI antibody was first detected.[2] By this method, the infection interval for sentinel birds in 1956 was 1 to 5 weeks longer than by viremia tests (Table 4). Similarly, in 1954 and 1955, initial infections of free-living birds estimated from HI antibody tests occurred during the last half of July, 1 to 3 weeks earlier than the first isolation of virus.

TABLE 4

*JE virus infection of sentinel BCNH in mosquito traps 1956**

Study site and traps	Evidence of infection†	Incidence for weeks beginning								Number of different birds		
		August					September					
		3	10	17	24	31	7	14	21	Tested	with viremia	developing HI antibody
Shinhama, 8 small traps on ground	Viremia	0/8	0/8	1/6	3/12	3/9	0/9	0/13	—	31	7	
	HI antibody	0/16	0/16	3/16	4/16	7/16	7/16	0/13	—	31		21
Sagiyama, 8 small traps & 1 Magoon trap on ground	Viremia	0/15	2/14	2/13	0/16	0/17	0/22	0/23	0/24	51	4	
	HI antibody	1/21	3/29	4/28	6/28	8/26	3/26	2/26	0/24	51		27

* Sentinel BCNH were tested weekly from 16 July to 17 September at Shinhama and 2 July to 24 September at Sagiyama; no evidence of infection was found before 3 August. All birds in traps were initially susceptible (*i.e.*, without neutralization or HI antibody) and infected birds were replaced by susceptibles within 2 days of the bleeding which first showed HI antibody.

† Fractions represent number of birds circulating virus on day of bleeding or showing development of HI antibody to titers of 1:10 or greater/number of birds tested per week.

TABLE 5

Incidences of avian JE virus infection at Shinhama and Sagiyama, 1954–1956

Manifestation of infection	Study site	Wild birds	Incidences before and after approximate onset of widespread virus dissemination*					
			1954		1955		1956	
			Before	After	Before	After	Before	After
HI antibody†	Shinhama	BCNH	2/59 (.03)	8/57 (.14)	—	42/90 (.47)	0/77	39/175 (.22)
		PE	0/25	6/32 (.19)	—	4/40 (.10)	0/72	3/57 (.05)
		LE	0/24	4/26 (.15)	—	3/28 (.11)	0/93	1/131 (.01)
	Sagiyama	BCNH	4/24 (.16)	2/8 (.25)	0/34	4/9 (.44)	0/44	4/50 (.08)
		PE	0/30	6/83 (.07)	0/32	15/94 (.16)	0/117	7/231 (.03)
		LE	1/30 (.03)	5/31 (.16)	0/5	2/23 (.09)	—	—
Virus‡ isolation	Shinhama	BCNH	0/55	2/105 (.02)	—	17/194 (.09)	0/34	7/90 (.08)
		PE	0/24	1/52 (.02)	—	1/54 (.02)	0/5	1/5
		LE	0/23	0/55	—	1/41 (.04)	0/39	2/94 (.01)
	Sagiyama	BCNH	0/27	1/40 (.03)	0/5	1/28 (.04)	0/25	3/36 (.08)
		PE	0/38	3/157 (.02)	0/33	8/217 (.04)	1/49 (.02)§	0/128
		LE	0/24	0/62	0/3	0/43	—	—

* Approximate onsets of widespread virus dissemination were 5 August 1954, 22 July 1955 and 29 July 1956 as determined by times of virus isolation and appearance of HI antibody in birds.

† Numbers of birds with HI titer of 1:10 or greater/numbers of birds tested. All birds were over 15 days of age to avoid maternal HI antibody.

‡ Numbers of plasmas from birds 6–70 days of age that yielded virus/numbers tested.

§ This virus was recovered on 25 July 1956, only 4 days before the date selected to represent the onset of widespread virus dissemination among birds.

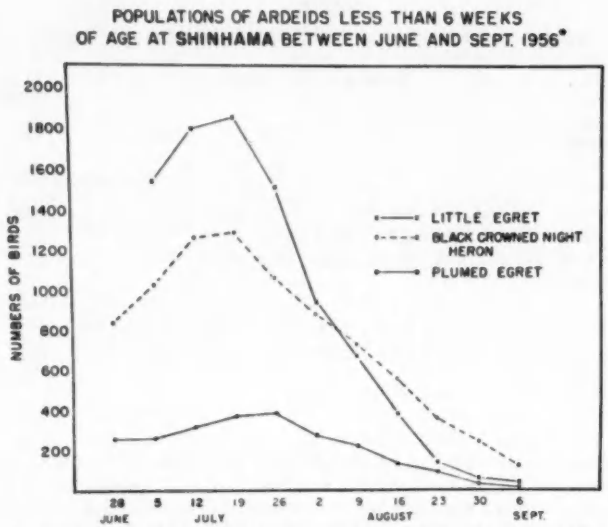

POPULATIONS OF ARDEIDS LESS THAN 6 WEEKS
OF AGE AT SHINHAMA BETWEEN JUNE AND SEPT. 1956*
NUMBERS OF BIRDS
2000
1800
1600
1400
1200
1000
800
600
400
200
LITTLE EGRET
BLACK CROWNED NIGHT HERON
PLUMED EGRET
28 JUNE
5
12
19
26
JULY
2
9
16
23
30
AUGUST
6
SEPT.
* BASED UPON THE ASSUMPTION THAT YOUNG ARDEIDS REMAIN CLOSE TO THE COLONY FOR 6 WEEKS AFTER HATCHING. THE VALUE FOR 28 JUNE EQUALS ALL BIRDS OF 1956 BROODS COUNTED THAT DATE (MOSTLY 0-4 WEEKS OF AGE), FOR 5 AND 12 JULY, THE NUMBERS OF NEW BIRDS 0-1 WEEK OF AGE WERE ADDED CUMULATIVELY. THE VALUES FOR 19 AND 26 JULY ARE SUMMATIONS OF BIRDS IN THE 0-3 OR 0-2 WEEK AGE GROUPS COUNTED ON 28 JUNE AND BIRDS 0-1 WEEK OF AGE COUNTED DURING THE 3 OR 4 WEEKS THEREAFTER. AFTER 26 JULY, THE FIGURES REPRESENT TOTALS OF 0-1 WEEK OLD BIRDS COUNTED DURING THE PREVIOUS 6 WEEKS.

FIG. 7

Incidence of avian infection at Shinhama and Sagiyama. Just as time of infection in sentinel birds was more accurately determined by serologic study than by virus isolation, so also was incidence of infection. Thus, during August and September 1956, when both methods were compared for sentinel BCNH during the period of virus dissemination among birds, greater numbers developed HI antibody (48/82) than showed detectable viremia (11/82) (Table 4). Similarly, demonstration of HI antibody was as much as 10-fold more sensitive for measuring infection incidence in free-living birds than was detection of viremia (Table 5).

In analyzing incidence of natural avian infection, birds tested before virus dissemination began were separated from those tested afterward. Obviously, the infection frequency in birds tested before virus dissemination would be zero, and inclusion of them with birds tested during August and September would reduce the overall incidence to an incorrectly low level. Thus, in Table 5, data for the periods before and after onset of widespread virus dissemination are presented separately. This analysis emphasizes that little, if any, avian infection occurred before late July–early August, 1954–56, and provides realistic incidences of avian infection with JE virus during the epizootic period. Incidences of infection as determined by HI antibody test varied from year to year and with species being consistently higher in BCNH than PE or LE. It should be noted that more virus strains were recovered from PE at Sagiyama in 1954 and 1955 (Tables 2 and 5), not because they were more frequently infected, for their infection rates were often less than those of BCNH (Table 5), but because they were more prevalent and accessible and thus were tested in larger numbers. A similar sampling bias did not occur at Shinhama since, in 1956, the proportion of BCNH to other birds bled after 29 July (Table 5) was almost identical with the actual proportion of BCNH in the total population during that period (Fig. 7), (175/363, .48 and 2600/5500 .47, respectively). Surprisingly, the ratios of tested PE and LE to total sample (57/363, .16 and 131/363, .36) were identical with their proportionate representation in the heronry (for PE 900/5500, .16; for LE, 2000/5500, .36).

Weekly variations in infection frequency were studied only in 1956 by testing sentinel BCNH in mosquito traps at Shinhama and Sagiyama (Table 4). Since infected birds were replaced with new susceptibles within 2 days of demonstration of HI antibody, the incidence figures in Table 4 represent *infection rates per week*. These frequencies tended to gradual rise and fall during the period of virus dissemination among birds. Thus, at Shinhama between 10 August and 7 September, infection frequencies based upon HI antibody tests increased from .00 (0/16) to .44 (7/16), after which no further infection was observed. At Sagiyama after a similar increase (.05 to .31) during the same period, infection frequencies fell to .00 during the next 2 weeks. This change in weekly infection frequencies tended to parallel the density of infected mosquitoes observed simultaneously in the same habitats.[4]

Time and incidence of avian infection at Tado and Unoyama. In 1955 an attempt was made to determine whether ardeid infection occurred elsewhere in Japan, outside the Tokyo area. Accordingly ardeids and cormorants at Tado and Unoyama, two heronries near Nagoya, 150 miles west-southwest of Tokyo (Fig. 1, article I), were studied for the development of HI antibody during July–September 1955. Serologic evidence of recent infection was obtained at approximately the same time, and in similar frequencies, as near Tokyo (Table 6). Five of 42 nestling cormorants studied at Unoyama possessed HI antibody to indicate recent infection during July and August 1955, assuming these colonies contained only JE virus like those near Tokyo.[14]

Infection of other birds. As pointed out in the first article of this series, ardeids were studied because they were numerous in the Kanto plain, were colonial, could be caught as nestlings in large numbers and were large enough to withstand repeated bleedings of 2 to 5 ml. Their selection did not imply that other birds are not important in the ecology of JE virus. However, except for cormorants at Daiganji in 1953[3] and near Nagoya in 1955, efforts made to implicate other species were unsuccessful. For example, during 1953–1956 no evidence was obtained for infection of young Blue Magpies, of which 49% possessed neutralizing antibody during 1950–1951,[8] JE virus was not recovered from 251 magpie bloods (Table 2) and tests for HI antibody in magpies over 15 days of age were negative (0/16 before and 0/7 after 22 July '55, and 0/29 after 29 July '56). Neutralizing antibody in magpies of similar age was not found in 1955 (0/12 before and 0/5 after 22 July), although in 1954 two of 59 birds were recorded as negative in the first plasma and positive in the second by the qualitative N test described elsewhere.[3] Maternal antibody to JE virus was not found in 23 magpies under 16 days of age in 1955 or in 115 in 1956, although in 1954, eight of 59 were recorded as having N antibody at 7 to 18 days of age, which subsequently became undetectable. Thus, aside from the observations in 1954, no evidence of infection of magpies with JE virus was obtained, and it seems justified to conclude that during 1953–1956 Blue Magpies in Irumagun did not play an important role in dissemination of JE virus.

TABLE 6

*Incidence of HI antibody in birds 16–70 days of age in the Tokyo and Nagoya areas of Japan 1955**

Study site	HI antibody incidence before and after approximate onset of widespread virus dissemination†	
	Before	After
Tokyo area		
Shinhama...	—	49/158 (.33)
Sagiyama....	0/71	21/126 (.17)
Nagoya area		
Tado.....	0/15	8/110 (.07)‡
Unoyama....	2/43 (.05)	10/27 (.37)

* BCNH, LE and PE were at Shinhama, Sagiyama and Tado, and BCNH and Cormorants at Unoyama.

† Approximate onset of widespread virus dissemination was 22 July 1955 in the Tokyo area. For this table the same date was used for Nagoya.

‡ In addition at Tado after 22 July, 2/6 Cattle Egrets and 0/4 Great Egrets had HI antibody.

DISCUSSION

This study establishes that ardeids at Shinhama and Sagiyama were regularly and silently infected with JE virus during the summers of 1952–1956. Experiment has shown that the viremia which almost invariably follows avian infection is of sufficient titer and duration[2] to infect colonized *C. tritaeniorhynchus*.[18] Further, in-

fected *C. tritaeniorhynchus* can transmit JE virus to birds, either experimentally[10] or in nature since, for practical purposes, the only infected arthropods recovered from the traps housing sentinel BCNH (Table 4) were *C. tritaeniorhynchus*.[4] It therefore follows that a bird-mosquito-bird infection cycle occurs in Shinhama and Sagiyama, and is responsible for the vast majority of avian infections. This is further supported by the observation that avian infection was not detected during spring and early summer, but rather appeared in mid-July, or early August, coincident with mosquito infection,[4] and persisted 4 to 6 weeks.

Since study of avian infection was necessarily confined to nestlings and juveniles, it was impossible to test adequate samples of birds beyond the first week in September. Therefore, although the *onset* of avian infection was sharply and unequivocally timed by sudden appearance of viremia and HI antibody in birds, the *end* of avian infection could not be determined. Since the susceptible avian population is never exhausted, avian infection probably continues until the vector disappears about mid-October. The significance of late avian infection and ardeid emigration in September and October is discussed elsewhere.[15]

The frequency of infection among nestlings during the epizootic varied yearly from .05 to .50 even in the same study area; it also changed weekly, tending to gradual rise and fall during the period of virus dissemination. Further, it varied among three species of ardeids, being greater in BCNH than in PE or LE. This difference, of interest because all three species coexist under similar ecologic conditions, is best explained by the zootropism of *C. tritaeniorhynchus*, which is attracted to BCNH more frequently than to PE or LE.[16]

Throughout the study it was recognized that infection frequencies alone were of limited value in determining total amount of avian virus available to mosquitoes, since obviously total susceptible bird populations also had to be known. However, susceptible bird populations at Shinhama and Sagiyama were diffiult to measure for four reasons. First, the total population changed week by week, from immigration in spring, breeding and dispersion in summer and emigration in fall.[15] Hence weekly observations were required. Second, accurate visual counts of adults were impossible during daylight hours, since many adult birds were away from the colonies obtaining food and those in the colony were constantly flying about. Thus, only nestlings and flightless juveniles could be counted accurately. Third, susceptibility to infection *i.e.*, absence of detectable N antibody, could be determined only in nestling and juvenile birds which could be readily captured, and not in adults. Shooting, the only available method for capturing adults, forced emigration at Shinhama and was prohibited at the Sagiyama National Preserve. Fourth, an uneven horizontal and vertical distribution of different ardeid species at Sagiyama made it impossible to enumerate even nestlings at that colony. Thus, it was possible to count all nestlings only at Shinhama where nesting was within 6 feet of the ground, and in essentially one plane.

Nevertheless, recognizing these limitations, two efforts were made to enumerate susceptible bird populations. One was a monthly count of adult egrets from aerial photographs of Shinhama and Sagiyama during 1955 and 1956. The other was a weekly count of nestling and flightless juveniles at Shinhama during July–September 1956. Being white, egrets not under foliage could be readily seen in aerial photographs (Fig. 3, article V), but since they feed outside colonies during daylight, the numbers photographed were increased twofold to obtain total egrets. Because ground observations showed that approximately one-third of birds at each heronry were BCNH, not visible in aerial photographs, the egret totals were further increased by one-third to obtain total ardeid populations. By this method, 2,400 to 3,600 adults were present at Shinhama and 3,000 to 5,400 at Sagiyama during the peak populations of July 1955 and 1956. Although adults presumably become immune in increasing incidence with age, many probably remained susceptible each year, since during 1950 and 1952, 22, 77 and 15% of 18 adult BCNH, 18 PE and 12 LE collected by shooting at Shinhama were without detectable N antibody (data from H. E. McCl.) and during 1954–1956, 33 to 93% of nestlings were without detectable maternal neutralizing antibody[3] suggesting that many parent hens were free of antibody and susceptible. Thus, there were apparently as many as several thousand susceptible adult ardeids at each colony.

To estimate susceptible nestling populations, nestlings were counted weekly until they fledged approximately 3 weeks after hatching. These measurements, possible only at Shinhama, were

attempted in 1956. By learning from ground observations that newly fledged juveniles did not usually fly beyond the heronries until 6 weeks of age, it was possible to add the new nestlings counted each week to those counted during the previous 5 weeks to estimate weekly totals in the colony (Fig. 7). Since upon loss of maternal neutralizing antibody, birds become uniformly susceptible to virus,[3] all nestlings and juveniles were susceptible by 2 to 3 weeks of age. In fact, because only 7 to 67% of nestlings ever have maternal antibody, many were susceptible before 2 to 3 weeks of age. Thus, the resultant curve fairly accurately represents the nestling segment of the susceptible bird population at Shinhama in 1956. From Fig. 7, it is evident that a maximum of over 2,000, and a minimum of 300, susceptible nestlings and juveniles were available for infection with JE virus after mid-July when virus dissemination began among birds. By multiplying these populations by the average incidence of infection among nestlings of three species at Shinhama in 1956, (approximately .10, Table 5), it is apparent that at least tens to hundreds of ardeids were infected weekly in 1956 at Shinhama during the period of virus dissemination. Since the Sagiyama and Nagoya colonies (Tado and Unoyama) also contained 1,000 to 5,000 birds, and since incidences of nestling infection there were similar to Shinhama, it is reasonable to conclude that additional tens to hundreds of nestling ardeids were also infected weekly at those colonies. While no information on infection of free-living adults and flying juveniles was obtained in this study, the fact that many are susceptible, are very likely bitten by *C. tritaeniorhynchus*[16] and circulate JE virus upon natural infection,[15] suggests that they also add to the pool of avian infection. The particular significance of infection in these birds to the local dissemination of JE virus is discussed elsewhere.[6, 15]

The time sequence of the bird population curve shown in Figure 7 is also important. Although for this curve to show total susceptible ardeid populations its ordinates would have to be increased by a constant number of susceptible adults, the shape of the curve accurately reflects increases in susceptible birds up to July when susceptibles begin to be converted into immunes by infection. By comparison of this curve with that of mosquito populations,[4] it can be seen that the peaks coincide chronologically. This observation suggests that bird and mosquito infection at the heronries are mutually interdependent, a conclusion supported by the absence of other possible vectors (ticks, mites, biting flies). For other bird species whose peak nestling populations occur in May or June, *e.g.*, CE, GE[15] and certain passerines which subsequently disperse from rural paddy areas of high vector-mosquito density to urban or mountainous regions, one would expect infection to be less frequent. Hammon's finding[8] of low antibody incidences in crows, starlings, martins and warblers is compatible with this thesis. Temporal differences among the curves of the three ardeid species are also noteworthy for it is evident that the prolonged breeding of BCNH furnished new susceptibles 1 to 2 weeks longer in August than LE or PE (Fig. 7). This may have contributed to the higher infection frequencies in BCNH than in LE or PE. Thus size, growth rate, time and duration of peak nestling populations play important roles in dissemination of JE virus in heronries.

However, extrapolation of these observations to other bird species in an attempt to enlarge the pool of virus available in viremic birds for infection of mosquitoes is not warranted, even in the Tokyo area. While the high incidence of infection in BCNH suggested by antibody studies in 1950[8] was confirmed in these studies, another species (Blue Magpies) with high incidence of N antibody after collection by shooting in 1950, was found to be infrequently infected and unimportant in the ecology of JE virus near Tokyo. Further, Blue Magpies and another species suggested by Hammon's survey as frequently infected (Dusky Thrush) did not regularly attract the vector mosquito, *C. tritaeniorhynchus*, at Shinhama.[16] It is therefore impossible to state with confidence the extent to which birds other than ardeids are important in the ecology of JE virus in Japan. Assuming that the "antibody" measured in serologic survey of birds is specific, as is suggested by Hammon's study,[8] avian infection would appear to involve multiple species in Japan and be of reasonably great density. However, even if this is true, one cannot assume that different birds are equally efficient in disseminating virus rapidly enough to mosquitoes to cause human epidemics.

SUMMARY

Infection with JE virus was a regular late-summer phenomenon among nestlings at two ardeid colonies near Tokyo. Avian infection

demonstrable by recovery of virus or appearance of antibody was first found in late July and early August of each year during 1952–1956 and was, for practical purposes, coincident with the demonstration of JE virus in mosquitoes. Frequencies of avian infection during virus dissemination (as measured by HI antibody) ranged from .01 to .47, being consistently greater in Black-crowned Night Herons than in Plumed or Little Egrets. Ardeid infection in two colonies near Nagoya in 1955 was similar in incidence and timing to that near Tokyo. Except for cormorants, no clear-cut evidence for infection of other bird species was obtained, and while serologic survey of Japanese wild birds suggests widespread involvement, the relative efficiency of other bird species for dissemination of JE virus is unknown.

ACKNOWLEDGEMENT

We are indebted to Drs. C. M. Southam and L. J. Kutner for supporting continued observations of avian infection at Shinhama and Sagiyama in 1954.

REFERENCES

1. Bawell, M. B., Deul, R. E., Jr., Matumoto, M., and Sabin, A. B., 1950. Status and significance of inapparent infection with virus of Japanese B encephalitis in Japan in 1946. *Am. J. Hyg.*, **51:** 1–12.
2. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., and McClure, H. E., 1959a. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses of birds. *J. Immunol.*, **83:** 605–613.
3. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., Kutner, L. J., and McClure, H. E. 1959b. Immunologic studies of Japanese encephalitis virus in Japan. IV. Maternal antibody in birds. *J. Immunol.*, **83:** 614–619.
4. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., Gresser, I., Hardy, J. L., and Bullock, H. R., 1959c. Ecologic studies of Japanese encephalitis virus in Japan. II. Mosquito infection. *Am. J. Trop. Med. & Hyg.*, **8:** 651–664.
5. Buescher, E. L., Scherer, W. F., Grossberg, S. E., Chanock, R. M., and Philpot, V. B., Jr., 1959d. Immunologic studies of Japanese encephalitis virus in Japan. I. Antibody responses following overt infection of man. *J. Immunol.*, **83:** 582–593.
6. Buescher, E. L., and Scherer, W. F., 1959e. Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemiologic correlations and conclusions. *Am. J. Trop. Med. & Hyg.*, **8:** 719–722.
7. Hammon, W. McD., Reeves, W. C., and Sather, G. E., 1951. Japanese B encephalitis virus in the blood of experimentally inoculated birds: epidemiological implications. *Am. J. Hyg.*, **53:** 249–261.
8. Hammon, W. McD., Sather, G. E., and McClure, H. E., 1958. Serologic survey of Japanese B encephalitis virus infection in birds in Japan. *Am. J. Hyg.*, **67:** 118–133.
9. McClure, H. E., Yoshii, M., Okada, Y., and Scherer, W. F., 1959. A method for determining age of nestling ardeids in Japan. *Condor*, **61:** 30–37.
10. Kitaoka, M., Okubo, K., Miura, T., and Nakamura, Y., 1953. Relationship between Japanese B and Russian Spring-Summer encephalitis and birds. *Jap. J. Med. Sc. and Biol.*, **6:** 247–259.
11. Mitamura, T., Kitaoka, M., Kobayashi, E., Okubo, K., and Tenjin, S., 1938. Evidence for inapparent infection with Japanese B encephalitis virus in so-called normal persons and animals. Abortive form of Japanese B encephalitis. *Tokyo Iji Shinshi*, **62:** 779–789. (in Japanese)
12. Mitamura, T., and Kitaoka, M., 1947. On the epidemiology of epidemic encephalitis. *The 12th Nihon Igakkai Kaishi*, 47–68. (in Japanese)
13. Sabin, A. B., Ginder, D. R., and Matumoto, M., 1947. Difference in dissemination of the virus of Japanese B encephalitis among domestic animals and human beings in Japan. *Am. J. Hyg.*, **46:** 341–355.
14. Scherer, W. F., and Buescher, E. L., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. *Am. J. Trop. Med. & Hyg.*, **8:** 644–650.
15. Scherer, W. F., Buescher, E. L., and McClure, H. E., 1959b. Ecologic studies of Japanese encephalitis virus in Japan. V. Avian factors. *Am. J. Trop. Med. & Hyg.*, **8:** 689–697.
16. Scherer, W. F., Buescher, E. L., Flemings, M. B., Noguchi, A., and Scanlon, J., 1959c. Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors; zootropism and vertical flight of *Culex tritaeniorhynchus*, with observations on variations in collections from animal-baited traps in different habitats. *Am. J. Trop. Med. & Hyg.*, **8:** 665–677.
17. Smorodintzeff, A. A., 1942. Progress of Soviet microbiologists in the study of various types of virus encephalitis and influenza. *A. Mikrob. Epid. Immun., Moskova*, **11-12:** 61–68. (in Russian)
18. Gresser, I., Hardy, J. L., Hu, S. M. K., and Scherer, W. F., 1958. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles, from infected pigs and chicks to susceptible pigs and birds. *Am. J. Trop. Med. & Hyg.*, **7:** 365–372.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN. V. AVIAN FACTORS

W. F. SCHERER, E. L. BUESCHER AND H. E. McCLURE

Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

INTRODUCTION

The repeated isolation of Japanese encephalitis (JE) virus from ardeids near Tokyo during 1952–1956[1] established that birds were involved in the natural history of JE virus and prompted investigation of the influence of avian factors such as behavior, bionomics and population dynamics upon virus ecology. In this discussion ardeids in colonies will be considered separately from those outside heronries, since only the former were studied intensively for evidence of virus infection.

MATERIALS AND METHODS

Study sites and birds. With the exception of the helicopter route for counting egrets (see Fig. 2, Article I), the study sites and birds have been described.[1, 11]

Ardeid nesting at Sagiyama. Nesting in a large zelkova tree was observed in 1955 and 1956 to time breeding periods of PE, GE and BCNH. In 1956 three areas within Sagiyama were studied to determine whether nesting was uniform in distribution for different species. Area I was located near areas 11 and 12 (Fig. 4, Article I) and consisted of a clump of low bamboo surrounded by tall zelkova and live oak trees. Area II covered an area of 120 degrees at a radius of 50 feet from area 19 and included bamboo 25 feet high, tall zelkova and a cluster of small broad-leafed evergreens. Area III was behind the house adjacent to area 19. Weekly counts of nestlings, requiring about 4 hours, were done by the same men who enumerated nestlings at Shinhama in 1956.[1] Birds in area I were counted from the ground by one observer; the other person counted birds in area II from a tree, and nests in area III from the ground. Birds were classified as 1, 2, 3, or more than 3, weeks of age (fledglings) on the basis of body size and plumage.

Ardeid dispersion and migration. From January 1955 through December 1956, egrets visible from the air at Shinhama and Sagiyama and along a defined 250-mile route over the lower Kanto plain east and north of Tokyo (Fig. 2, Article I) were counted indirectly from magnified aerial photographs of the heronries or directly from a helicopter traveling about 60 miles per hour at 500 feet altitude. At this height egrets were visible up to one-half mile on either side of the helicopter. Trips were made between the 17th and 27th of each month starting at 8–9 a.m. The route was always flown in the same direction and was covered usually by mid-afternoon. Since counts were made during the morning and early afternoon when egrets feed in rice paddies, many egrets were extracolonial. Identification of egrets by species was impossible from the air even with binoculars; however, ground observations made while driving over portions of the helicopter route, established that the Little Egret was practically the only species present in the winter whereas Plumed Egrets were preponderant in the summer. Flocks were recorded in sizes of 1, 2, 3 to 5, 6 to 10, 11 to 20, 21 to 30, 31 to 50 or greater than 50 birds.

Tests for JE virus and maternal antibodies. These methods are presented elsewhere.[1, 3]

RESULTS AND DISCUSSION

Many phenomena involving ardeids conceivably influence dissemination of JE virus; among them are the following:

A. Intracolonial Factors

1. Innate immunity related to
   a. Species
   b. Age
2. Acquired immunity
   a. Maternal antibodies (passively acquired)
   b. Antibodies following infection (actively acquired)
3. Anatomical features
4. Nest habitats
5. Feeding habits
6. Flight activities
7. Breeding
   a. Age and yearly frequency

b. Seasons

B. Extracolonial Factors

1. Flight range during breeding season
2. Local dispersion in Fall
3. Migration

Limitations in study techniques made it difficult to evaluate each factor objectively. Furthermore, although quantitative studies of avian infection were confined to nestlings because older birds could not be readily captured, the role of adult birds in the natural history of JE virus cannot be ignored. Therefore it is with limitations that ardeid factors in dissemination of JE virus are discussed.

Intracolonial Ardeid Factors in the Ecology of JE Virus at the Shinhama and Sagiyama Heronries

Innate immunity related to species. There appeared to be no innate species resistance of ardeids to JE virus infection. Each species, BCNH, LE and PE was infected, circulated virus and developed antibodies in nature,[1] and although infection frequencies differed among species, the differences were explainable by other than innate resistance. For example, the higher incidence of recent natural infection in BCNH than in LE[1] could be explained by the tendency of *Culex tritaeniorhynchus* to bite BCNH more frequently than LE.[12]

Innate immunity related to age. Since frequency of viremia in chickens inoculated with JE virus varied with age,[2] age and maturity of ardeids were considered possible factors in virus ecology. However, experience with young sentinel BCNH in mosquito traps at Shinhama and Sagiyama in 1952 and 1956 showed that no resistance to infection developed during the first 4 months of life. For example, during August and September 1956 viremia occurred and hemagglutination-inhibition (HI) antibody developed in 11 BCNH between 9 and 13 weeks of age, and HI antibody developed without viremia detectable by weekly bleedings, in another 46 BCNH 9 to 16 weeks of age. That older ardeids could be infected naturally was shown in 1953, when on 10 and 16 August, viremia was detected in two sentinel BCNH 12 to 13 months old. Neutralizing (N) antibody appeared (LNI 2.1 or greater) in one bird 9 days after demonstration of viremia; unfortunately the other BCNH died on 10 August from hemorrhage at the site of venipuncture and could not be followed for antibody development. Thus, these observations established that BCNH do not become resistant during their first summer of life and can be infected during their second summer. Whether fully mature BCNH without previous infection by JE virus or egrets of any age are as susceptible as nestling and juvenile BCNH remains unknown.

Maternal, passively acquired antibodies and immunity. The temporary protective effect of maternal antibody in certain birds against infection by JE virus[3] made it important to measure the incidence of this antibody in wild ardeids and to evaluate its significance in virus ecology. Studies reported elsewhere established the superiority of the N test for detection of maternal antibody, its rate of decay, and the method for analysis of data as given in Table 1.[3] These studies also suggested that maternal N antibody in certain nestling ardeids at Shinhama and Sagiyama was the result of JE virus infection of hens while in others it was an antibody induced probably outside of Japan by infection with an immunologically related virus. Whereas the former antibody was protective, the latter probably was not. Since during these studies it was impossible to distinguish between these antibodies in individual birds, the incidence data presented represent total frequencies of both homologous and heterologous antibodies.

During 1953–1956 antibody that neutralized JE virus existed in 7 to 67% of newly hatched birds less than 16 days of age at Shinhama and Sagiyama (Table 1). Higher frequencies were usually found in BCNH and PE than in LE, and maternal antibody was present in nestling ardeids hatched throughout the entire May–August breeding season (Table 2). Variations in incidence were observed from year to year, and month to month. Although these variations reflect differences in immune status of hens producing nestlings at different times, it is unknown whether the same hens returned to a colony to breed each year and whether the hens breeding in May and June were the same or different from those breeding in July and August. Had protective maternal antibody been more frequent during May and June than during July–September, it might have prevented dissemination of virus among nestlings before late July and explained the time sequence of avian epizootics.[1] However, monthly incidences of maternal antibody (Table

TABLE 1

Yearly occurrence of maternal antibody in birds less than 16 days of age

Bird	Year	No. birds with neutralizing antibody in plasma/no. birds tested			
		Shinhama		Sagiyama	
		Minimum incidence*	Maximum incidence†	Minimum incidence	Maximum incidence
BCNH	1953	36/69 (.52)	46/69 (.67)		
	1954	63/168 (.38)		37/60 (.62)	
	1955	26/104 (.25)		35/88 (.40)	
	1956	119/301 (.40)	189/301 (.63)	42/135 (.31)	86/135 (.64)
LE	1953	10/39 (.26)	25/42 (.60)		
	1954	9/121 (.07)		13/62 (.21)	
	1955	0/30		8/79 (.10)	
	1956	10/124 (.08)	30/124 (.24)		
PE	1953	3/12 (.25)	5/13 (.38)		
	1954	19/57 (.33)		15/50 (.29)	
	1955	5/23 (.22)		46/144 (.32)	
	1956	28/144 (.25)	57/144 (.50)	37/135 (.27)	63/135 (.47)
CE	1954			4/14 (.29)	
	1955			13/45 (.29)	
	1956			18/56 (.32)	26/56 (.46)
GE	1954	13/35 (.37)		22/57 (.40)	
	1955			19/33 (.60)	

* Minimum incidence = for 1953 & 1956, birds with LNI greater than 1.7/no. tested and for 1954 & 1955, birds with qualitative neutralization test positive/(no. tested minus those with "equivocal results").

† Maximum incidence = for 1953 and 1956, birds with LNI greater than 0.9/no. tested.

2) were not sufficiently different consistently enough to support this hypothesis.

Theoretically, maternal antibody induced in hens by JE (and not another group B arthropod-borne) virus might have three effects upon natural infection in individual nestlings. 1) It might protect a bird from infection until it fledged, after which its greater activity and plumage might reduce the effectiveness of biting mosquitoes and thus prevent the bite of an infected mosquito. Such a nestling would escape virus infection. 2) It might prevent viremia even though the bird became infected and developed antibodies. 3) It might merely delay infection until N antibody disappeared at 1 to 3 weeks of age and the bird became fully susceptible. The first two effects of maternal antibody obviously would be important factors in dissemination of JE virus since they would reduce the incidence of viremia in nestlings and result in less frequent transfer of virus from bird to mosquito. However, the third possibility seems most probable since, after loss of maternal antibody, birds in the laboratory and in mosquito traps in nature readily become infected and viremia occurs.[1, 2] In the over-all ecology of JE virus a 1 to 3-week delay in infection of 7 to 67% of nestlings probably does not significantly alter their role in seeding mosquitoes with JE virus during the encephalitis season.

Nevertheless, in 1953 initial consideration of the possible influence of maternal antibody on frequencies of detectable viremia in nestlings, led to the policy of attempting virus isolations only from birds over 6 days of age.[1] Although birds beyond 3 to 4 weeks of age would have been uniformly without maternal antibody,[3] ardeids fledge at that time and can not be readily caught. Whether the decision to test only birds over 6 days of age for viremia actually increased the number of strains recovered is now difficult to

TABLE 2

Monthly occurrence of maternal antibody in birds less than 16 days of age during breeding seasons of 1953–1956

Birds and month of hatching	No. birds with neutralizing antibody in plasma/no. birds tested*						
	Shinhama				Sagiyama		
	1953	1954	1955	1956	1954	1955	1956
BCNH:							
May	10/27 (.37)	11/27 (.41)		12/40 (.30)	17/22 (.77)	12/22 (.54)	2/20 (.10)
June	17/24 (.71)	21/49 (.43)		63/140 (.45)	7/12 (.58)	15/42 (.36)	17/74 (.23)
July	7/13 (.54)	27/69 (.39)	12/69 (.17)	36/104 (.35)	11/24 (.46)	6/21 (.29)	21/39 (.54)
Aug	2/5 (.40)	4/23 (.17)	14/35 (.40)	8/17 (.47)	2/2	2/3 (.67)	2/2
LE:							
May	7/21 (.33)	3/22 (.09)			1/4 (.25)	0/2	
June	3/18 (.17)	4/32 (.13)		7/72 (.10)	11/35 (.31)	7/53 (.13)	
July	0/3	2/41 (.05)	0/25	3/45 (.07)	1/14 (.07)	1/22 (.05)	
Aug		0/16	0/5	0/7	0/9	0/2	
PE:							
May				0/5			
June	1/8 (.13)	14/30 (.47)		18/65 (.28)	10/25 (.40)	32/61 (.52)	17/75 (.23)
July	1/4 (.25)	5/21 (.24)	5/22 (.23)	9/39 (.23)	5/18 (.28)	12/70 (.17)	19/55 (.36)
Aug	1/1	0/6	0/1	1/5 (.20)	0/7	2/13 (.15)	1/5 (.20)

* For 1953 and 1956, birds with LNI greater than 1.7/no. tested; for 1954 and 1955 birds with qualitative neutralization test positive/(no. tested minus those with "equivocal" results).

determine, for tests for maternal antibody and virus were not done simultaneously on birds younger and older than 6 days. The only available data that relate presence of maternal antibody to absence of viremia concern BCNH from different years (Table 3). In 1953, a high incidence of maternal antibody was associated with failure to detect viremia and in 1955 a lower maternal antibody incidence coincided with recovery of virus. However, in 1956, perhaps due to a high density of infected mosquitoes, the association was poor since virus was recovered from six of 34 BCNH, despite a high incidence (.50) of maternal antibody. Thus, there are no data to demonstrate conclusively that incidence of maternal antibody actually influences frequency of virus recovery from 0 to 3-week-old birds, although this is a reasonable assumption if the antibody is specific for JE virus and protective to the bird.

Antibodies and immunity acquired from infection. Actively acquired antibodies and immunity that follow natural infection of ardeids by JE virus obviously play an important role in ecology of JE virus at heronries. To show that ardeids with N antibody fail to circulate virus upon reinoculation by needle and syringe, four BCNH infected in nature 2 to 2.5 months previously and four infected 13 to 14 months before, were challenged with $10^{7.7}$ weanling-mouse-LD_{50} of virus subcutaneously. Their log neutralization indices of plasma when inoculated ranged from 1.7 to 3.0 except for one bird which was 1.1. No viremia was detected by daily bleedings and intracranial inoculation of weanling mice, through day 7 or on day 10 even though each bird was given cortisone acetate, 10 mg/KBW intramuscularly on days −2, −1 and 0.[8] Similarly, none of 11 mature PE, experimentally infected 10 to 13 months previously, circulated JE virus on days 2 to 8 after intravenous inoculation of approximately 10^9 LD_{50} of virus.[6] Assuming no difference between experimental inoculation and infection by mosquito bite, it would seem that ardeids become infected and serve as sources of JE virus for mosquitoes only once in their lives. However, the recent observation made by Reeves *et al.*,[10] that Western equine encephalitis virus can be recovered from tissues of occasional birds

TABLE 3

*Relationships between frequencies of maternal neutralizing antibody and incidences of detectable viremia in BCNH over 10 days of age**

1953		1955		1956	
Maternal antibody†	Viremia†	Maternal antibody	Viremia	Maternal antibody	Viremia
10/18 (.55)	0/18	26/102 (.25)	6/102 (.06)	17/34 (.50)	6/34 (.18)

* These data are from the periods of known intracolonial virus dissemination in ardeids at Shinhama which began approximately 1 week prior to the first recovery of JE virus from an ardeid (26 July, 1953, 22 July, 1955 and 29 July, 1956) and extended until birds could no longer be captured in September.

† Numbers of plasmas positive for antibody or virus/numbers tested for both. Plasma was positive for antibody in 1953 and 1956 if the LNI was greater than 0.9, and in 1955 if the qualitative neutralization test was positive or equivocal.

infected months before suggests that the previous statement may not apply to all birds in a population or to all viruses. Nevertheless, it seems reasonable to conclude, for the time being at least, that infection by JE virus changes most susceptible ardeids into resistant hosts and thus limits yearly populations of susceptibles liable to become viremic and disseminate virus to mosquitoes.

Anatomical features of ardeids. The skin of nestling ardeids up to 2 or 3 weeks of age is thin and soft, and is covered only by loose fluffy down which is no impediment to biting mosquitoes. After 3 to 4 weeks of age, however, feathers cover the skin except on the legs and near the eyes, and offer some degree of protection from mosquito bites. Unfortunately, relationships of anatomy to mosquito exposure are difficult to study quantitatively in nature; therefore one can only call attention here to the possibility that anatomical features of ardeids play a part in transfer of virus between mosquitoes and birds. Perhaps some insight into the influence of bird anatomy upon biting by vector mosquitoes can now be obtained with colonized *C. tritaeniorhynchus*.[9]

Nest habitats of ardeids. The accessibility of nestlings to mosquitoes was governed in part by nest habitats. For example, both the incidence of bird infection and density of mosquitoes, were less in trees 24 to 50 feet above ground at Sagiyama than at ground level,[12] and altitude of nests apparently limited nestling infection rates. This was less important at Shinhama where most nests were 4 to 8 feet above ground in bamboo thickets, than at Sagiyama where nests were very unevenly distributed vertically. For example, BCNH nests at Sagiyama were much more plentiful in one area 25 to 60 feet high (area III, Fig. 1) than in two other portions of Sagiyama where nests were closer to the ground (areas I and II). Also, there was an uneven horizontal distribution of nestling populations of different species at Sagiyama (*e.g.*, CE, GE and PE, Fig. 2) which created, within a single macrohabitat, different microhabitats that in turn affected mosquitoes and spread of virus.[12]

Feeding habits of ardeids. Ardeid nestlings are fed by regurgitation of fish, loaches, crustacea and frogs from parents' stomachs or mouths. Feeding begins soon after hatching and continues many times daily until the two or three young in each nest fledge at 3 to 4 weeks of age. Since this mechanism affords opportunity for infestation of nestlings by endoparasites from adults or food, the possibility of virus transfer by endoparasites had to be considered. Indeed, two nematodes,

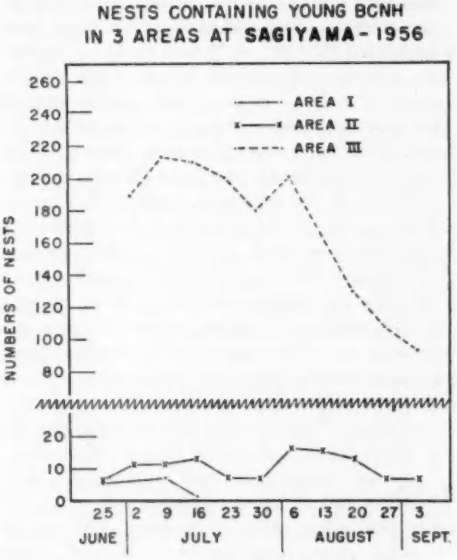


FIG. 1

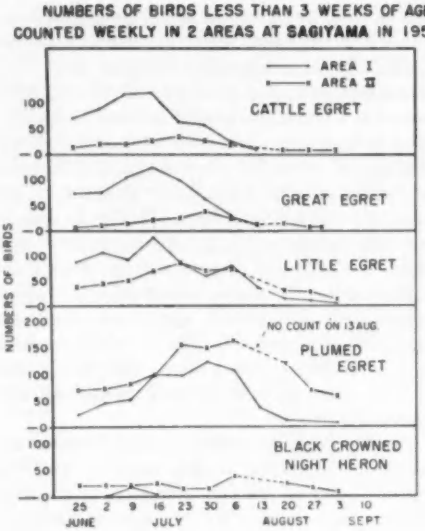

NUMBERS OF BIRDS LESS THAN 3 WEEKS OF AGE
COUNTED WEEKLY IN 2 AREAS AT SAGIYAMA IN 1956
AREA I
AREA II
CATTLE EGRET
GREAT EGRET
LITTLE EGRET
NO COUNT ON 13 AUG.
PLUMED EGRET
BLACK CROWNED NIGHT HERON
NUMBERS OF BIRDS
25 2 9 16 23 30 6 13 20 27 3 10
JUNE JULY AUGUST SEPT

FIG. 2

Eustrongylides mergorum and *Contracaeca microcephalum* frequently attached to the wall of the lower esophagus and stomach of ardeid nestlings soon after hatching. They were frequently filled with blood, and by 3 or more weeks of age, had burrowed deeply into the stomach wall. In gaining access to blood and tissues they could have inoculated JE virus, or in turn could be infected by ingestion of viremic blood. While purely speculative at the moment, this possible method of virus transmission is worthy of future investigation and emphasizes that avian feeding habits must be considered in studies of JE virus ecology.

Flight activities of ardeids. Adult BCNH feed at night; egrets, on the other hand, feed during the day. Thus, at dusk and dawn, the principal periods of vector mosquito biting activity, egret nestlings are usually covered by hens whereas BCNH nestlings are alone in nests. Further, the presence of adults may reduce mosquito attack rates on nestlings simply by attracting mosquitoes to them rather than nestlings. These behavior patterns may have contributed to the higher infection frequency of BCNH than of PE or LE.[1]

Age and frequency of breeding of ardeids. Most BCNH breed during the third summer of life, by the time adult plumage develops. The age of breeding egrets could not be ascertained in the field since plumage changes were not obvious although several Little Egrets in aviary cages began breeding during the third summer after hatching. It was impossible to determine what proportion of birds at the colonies were breeding, the duration of their reproductive life, and whether hens had been in the colony and thus exposed to JE virus during previous years or were new and of totally unknown immune status. In fact, even the sex of ardeids could not be determined by inspection of the external anatomy. From observations of individual nests it seemed likely that the number of broods per female per season was not more than two, but whether it was usually one or two was difficult to determine. At the aviary in 1956, Little Egrets produced two clutches of eggs in one season.

Breeding seasons of ardeids. During the winter months only a few hundred LE lived at Shinhama, and at Sagiyama, ardeids were absent. Migration to both colonies occurred yearly in March and April (Fig. 3). Often birds would appear suddenly and immigration would be completed in a period of 1 to 2 weeks. Breeding began in April and terminated in September, but the periods for each ardeid species differed. Observations in 1954 and 1955 of Great Egrets (GE) nesting in a zelkova tree at Sagiyama established that they bred only during May, June and early July, and by late July and August, when JE virus was present in mosquitoes,[4] GE nestlings did not exist. The same observation applied to Cattle Egrets (CE); in fact, by mid-July many Cattle Egrets had left Sagiyama. The early breeding of Great and Cattle Egrets thus not only may have limited the numbers infected by JE virus, but prevented our studying them as nestlings during the period of virus dissemination. Contrariwise, the longer breeding periods of BCNH, PE and LE, *i.e.*, until mid-August provided nestlings concurrently with infected mosquitoes.[4]

Extra-colonial Ardeid Factors in the Ecology of JE Virus

Since ardeids constantly move between colonies and surrounding paddyland to obtain food, their extracolonial activities need to be considered in this ecologic evaluation. Only three extracolonial activities are discussed, although others may prove to be equally important.

Flight range during breeding season. Adult ardeids fly daily up to 20 miles from the colonies to obtain food. This was evidenced for example,

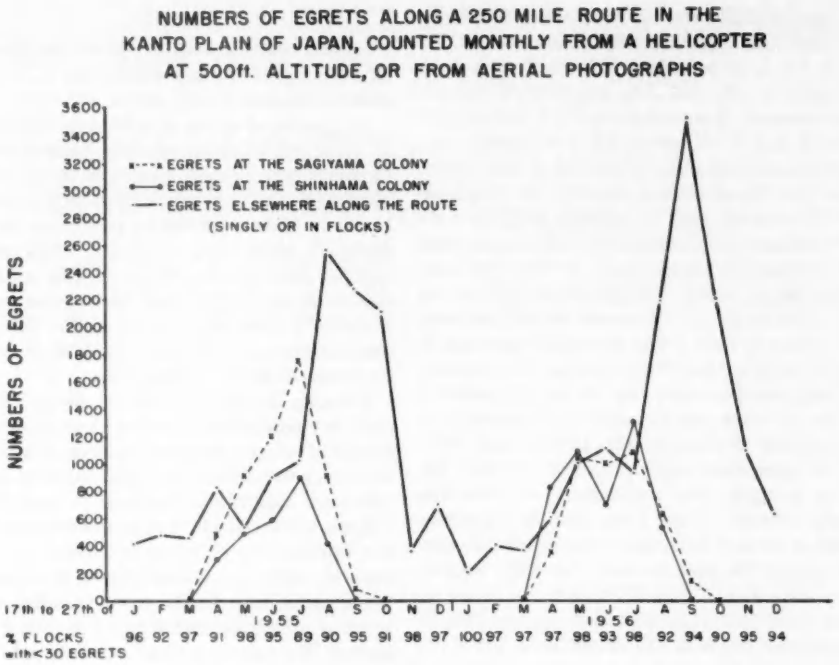

NUMBERS OF EGRETS ALONG A 250 MILE ROUTE IN THE KANTO PLAIN OF JAPAN, COUNTED MONTHLY FROM A HELICOPTER AT 500ft. ALTITUDE, OR FROM AERIAL PHOTOGRAPHS
NUMBERS OF EGRETS
EGRETS AT THE SAGIYAMA COLONY
EGRETS AT THE SHINHAMA COLONY
EGRETS ELSEWHERE ALONG THE ROUTE (SINGLY OR IN FLOCKS)
17th to 27th of
1955
1956
% FLOCKS with<30 EGRETS
96 92 97 91 98 95 89 90 95 91 98 97 100 97 97 97 98 93 98 92 94 90 95 94

FIG. 3

by the finding of salt water fish in egrets at Sagiyama, more than 15 miles from salt water. Thus it is possible that susceptible adults might become infected outside colonies and return to circulate virus and infect *C. tritaeniorhynchus* in colonies or vice versa. The frequency with which such movement of virus occurs is unknown, but since 22 to 77% of adult ardeids surveyed in 1950 were without N antibody (data from HE McClure) and since maternal neutralizing antibody was absent in 33 to 93% of nestlings (Table 1), it seems likely that numerous susceptible adults exist at Shinhama and Sagiyama to transfer virus daily up to 20 miles in and out of the colonies.

Local dispersion in Fall. As breeding ceases and young, mature ardeids move permanently from colonies during August–October. This emigration is depicted in Fig. 3 for 1955 and 1956 by decreasing numbers of intracolonial egrets and by increases in egrets elsewhere on the Kanto plain. Most egrets outside the colonies of the Kanto plain probably come from local heronries, although some may migrate from more northern sections of Japan. That this increase in egret population outside heronries actually represents dispersion is emphasized by the fact that the distribution of flock sizes remains constant despite an increase in total population. Since this dispersion occurs during the period of extensive avian infection at the colonies,[1] it is probable that viremic ardeids transfer JE virus to other foci in the Kanto plain where, if *C. tritaeniorhynchus* exist, further dissemination of virus to other vertebrates, including man, is possible. A fall dispersal of viremic ardeids, together with the 1 to 2-week period between infection of mosquitoes and their ability to transmit,[7] help to explain continued occurrence of human disease into early October.[13]

Migration. The cyclic appearance of ardeids at Shinhama and Sagiyama in spring and their disappearance in fall (Fig. 3) are manifestations of migration. This migration from the Tokyo area could conceivably be a factor in terminating epidemics and epizootics of JE near Tokyo, although cessation of *C. tritaeniorhynchus* breeding is probably more important in this respect.

However, serious consideration must be given

to the possibility that JE virus is transferred long distances by migrating ardeids[5] since BCNH, PE and LE have a widespread distribution from tropical to temperate Asia, and migrate between summer and winter residences. Both directions of ardeid and virus movement are possible, *i.e.*, toward and away from the study sites near Tokyo. Yet the absence before mid-July of detectable avian infection, even by sensitive antibody tests of nestlings at Shinhama and Sagiyama, is hard to explain by introduction of virus by birds migrating to Japan 3 months earlier each spring from Southeast Asia. Moreover, virus introduced into colonies near Tokyo by adults migrating in April would not find *C. tritaeniorhynchus* available in large numbers until June for serial transfer of virus to other ardeids and maintenance of a population of viremic birds. In 1953 and 1957, when mosquitoes were examined carefully for virus in April, May and early June, none was found infected.[4] These facts, plus the finding in 1956 of virus in mosquitoes well before infection of birds in the same habitat,[1, 4] strongly suggest that birds do not bring JE virus to the heronries each year, but rather that virus appears first in mosquitoes which in turn infect birds. Yet it remains remotely possible that another biting ecto- or endoparasite which transfers virus only among *adult* birds, and whose infection is not detected by sentinel pigs or by pig- or bird-baited trap collections, transfers virus among *adult* ardeids during Spring without involvement of nestlings. Until methods become available to study adult birds intensively, this uncertainty cannot be resolved and the introduction of virus by immigrating ardeids firmly eliminated as a possibility.

On the other hand, the chance that ardeids carry virus south upon migration in the fall is excellent.[6] Migrating from the Kanto plain begins in early September and by November only a few widely scattered egrets can be seen (Fig. 3). Since migration begins while avian infection is still demonstrable at the colonies,[1] it is possible that some birds leave the Kanto plain circulating virus and infect mosquitoes elsewhere to the south. Because migration speed of ardeids is unknown, it is uncertain how far a bird infected near Tokyo might travel before viremia ceased. However, it is likely that the distance covered is hundreds, rather than thousands of miles.

SUMMARY

Certain characteristics of ardeids and their behavior were important in the ecology of JE virus at heronries near Tokyo, Japan.

No species of ardeid was innately immune to JE virus but, following infection, birds developed immunity which probably reduced the size of the susceptible bird population. Up to 67% of newly-hatched birds possessed passively-acquired, maternal, neutralizing antibody which subsequently disappeared. Although this antibody could temporarily delay infection of nestlings, it probably did not significantly reduce the total seasonal amount of JE virus available in ardeids for infection of vector mosquitoes.

Breeding habits governed the supply of susceptible nestlings which, when viremic, serve as sources of virus for vector mosquitoes. Anatomical features, nest habitats and flight habits of adults influenced exposure of nestlings to mosquitoes. Flight activities and feeding habits made possible the introduction of virus into heronries from without, either by viremic birds or by unknown mechanisms; for example, tissue invading endoparasites. Migration and dispersal of birds during August–November provided possible means of spreading virus beyond the colonies.

REFERENCES

1. BUESCHER, E. L., SCHERER, W. F., McCLURE, H. E., MOYER, J. T., ROSENBERG, M. Z., YOSHII, M., AND OKADA, Y., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. *Am. J. Trop. Med. & Hyg.*, **8**: 678–688.
2. BUESCHER, E. L., SCHERER, W. F., ROSENBERG, M. Z., AND McCLURE, H. E., 1959b. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses of birds. *J. Immunol.*, **83**: 605–613.
3. BUESCHER, E. L., SCHERER, W. F., ROSENBERG, M. Z., KUTNER, L. J., AND McCLURE, H. E., 1959c. Immunologic studies of Japanese encephalitis virus in Japan. IV. Maternal antibody in birds. *J. Immunol.*, **83**: 614–619.
4. BUESCHER, E. L., SCHERER, W. F., ROSENBERG, M. Z., GRESSER, I., HARDY, J. L., AND BULLOCK, H. R., 1959d. Ecologic studies of Japanese encephalitis virus in Japan. II. Mosquito infection. *Am. J. Trop. Med. & Hyg.*, **8**: 651–664.
5. BUESCHER, E. L., 1956. Arthropod-borne encephalitides in Japan and Southeast Asia. *Am. J. Pub. Health*, **46**: 597–600.
6. BYRNE, R. J., 1959. Personal communication.
7. GRESSER, I., HARDY, J. L., HU, S. M. K., AND SCHERER, W. F., 1958. Factors influencing

transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles, from infected pigs and chicks to susceptible pigs and birds. *Am. J. Trop. Med. and Hyg.*, **7**: 365–373.

8. GROSSBERG, S. E., 1959. Personal communication.
9. NEWSON, H. D., BLAKESLEE, T. E., TOSHIOKA, S., SAKAI, M., WHEELER, C. M., SHIMADA, T., AND AKIYAMA, J., 1956. A preliminary report on the laboratory colonization of the mosquito *Culex tritaeniorhynchus* Giles. *Mosq. News*, **16**: 282.
10. REEVES, W. C., HUTSON, G. A., BELLAMY, R. E., AND SCRIVANI, R. P., 1958. Chronic latent infections of birds with western equine encephalomyelitis virus. *Proc. Soc. Exp. Biol. and Med.*, **97**: 733–736.
11. SCHERER, W. F., AND BUESCHER, E. L., 1959. Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. *Am. J. Trop. Med. & Hyg.*, **8**: 644–650.
12. SCHERER, W. F., BUESCHER, E. L., FLEMINGS, M. B., NOGUCHI, A., AND SCANLON, J., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors. Zootropism and vertical flight of *Culex tritaeniorhynchus* with observations on variations in collections from animal baited traps in different habitats. *Am. J. Trop. Med. & Hyg.*, **8**: 665–677.
13. SCHERER, W. F., KITAOKA, M., OKUNO, T., AND OGATA, T., 1959b. Ecologic studies of Japanese encephalitis virus in Japan. VII. Human infection. *Am. J. Trop. Med. & Hyg.* **8**: 707–715.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN

VI. Swine Infection*

W. F. SCHERER, J. T. MOYER, T. IZUMI†, I. GRESSER AND J. McCOWN†

Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

INTRODUCTION

Although it was known from earlier surveys for neutralizing (N) antibody that inapparent infection of pigs was common in Japan,[1] prior to 1956 little emphasis was placed on their possible role in the yearly ecology of JE virus. In that year our attention became focused on the large swine population near Tokyo and its annual turnover, occasioned by high birth rate and intentional slaughter by 16 months of age. Together these factors produce each summer many susceptible pigs capable of circulating JE virus in blood[11] and infecting large numbers of vector mosquitoes.[5, 12] Recognition of the possible importance of swine in dissemination of JE virus near Tokyo was the stimulus which prompted these investigations of natural swine infection in 1956 and 1957. The studies were designed to learn frequency and time of swine infection in natural habitats, for ultimate correlation with similar information concerning man, birds and mosquitoes.[2]

MATERIALS AND METHODS

Study areas. Pigs were at three locations, Sagiyama, Tokyo and Zama in 1956 and at Sagiyama, Shinhama and Minami-gyotoku in 1957 (Fig. 2, Article I). Although swine were not normal inhabitants of the Shinhama and Minami-gyotoku areas and were therefore not studied there in 1956, they were employed in the Shinhama and Minami-gyotoku mosquito traps in 1957 because they attracted large numbers of the vector, *C. tritaeniorhynchus*.[12]

Locations of mosquito traps containing pigs at Sagiyama, Tokyo and Zama in 1956 and at Sagiyama, Shinhama and Minami-gyotoku (hereafter referred to merely as Shinhama) in 1957 are pictured and described in detail elsewhere.[3, 10, 12] In addition, in 1956 and 1957, pigs were studied on farms (Figs. 1–3) within 3 to 4 miles of the Sagiyama heronry and within 5 miles of the school-children study site at Kasukabe[13] (Fig. 4). In 1956, 71 pigs were fairly uniformly distributed over 26 farms; the 27th farm was a large one with over 100 pigs, 51 of which were bled in these studies. In 1957 pigs were on 64 farms (2 to 4 pigs per farm) in the same general areas as in 1956.

Pigs. Yorkshire and Berkshire swine on farms ranged in age from 2 to 7 months when first bled in May 1956 and April 1957; pigs from Hokkaido* in 1957 were 2 months old when first tested in April. Most pigs were weaned at about 40 days of age. The nine Yorkshire pigs used in mosquito traps in 1956 were from one litter born in mid-March at Irumagun (Fig. 2, Article I), and were weaned 2 days before their first bleeding on 9 May.

Collection of swine plasma. The methods used on farms in 1956 for bleeding pigs from the tails or ears have been presented elsewhere.[11] Femoral venipuncture with a syringe wet with heparin solution (1 mg per 0.1 ml) was often employed in 1956 for pigs at the laboratory and was uniformly used in 1957.

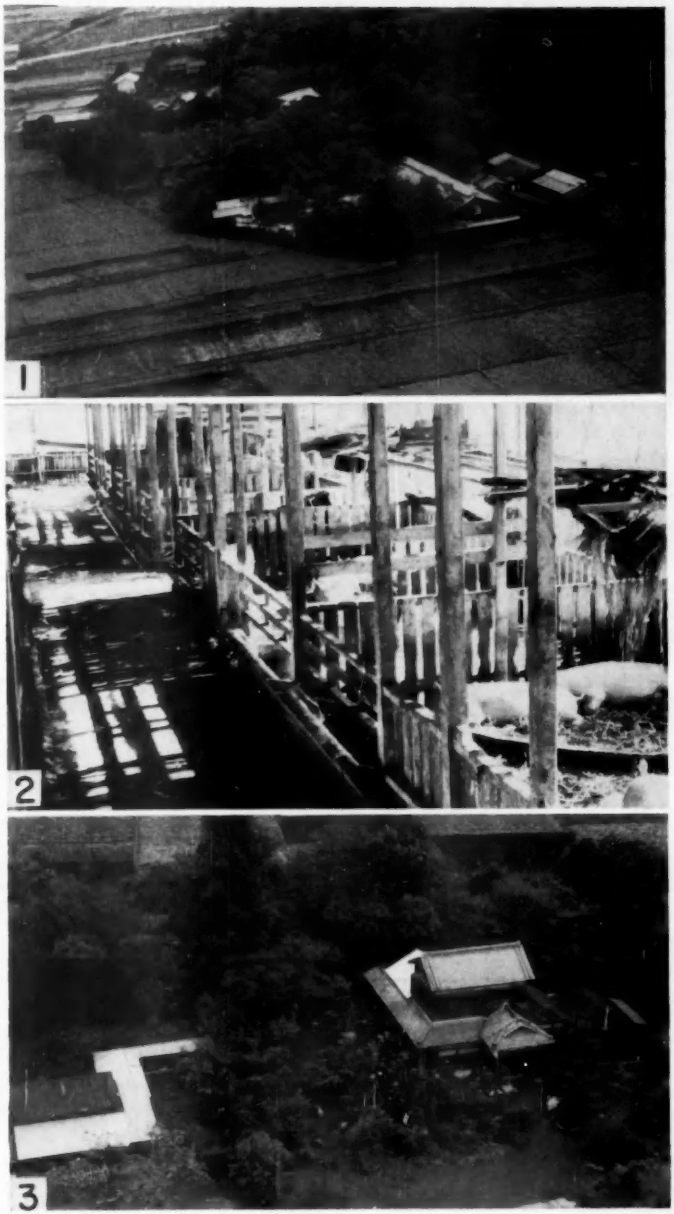
Determinations of antibodies in plasma. Antibodies were measured in 1956 by the N, hemagglutination-inhibition (HI) and complement fixation (CF) tests as reported.[11] In 1957 the techniques differed only in that the incubation period for N tests was 1 hour at 37°C and 1 hour at 4°C rather than 2 hours at 37°C, and the 12th mouse passage of JE virus, strain M1/311, was used instead of the 9th–11th.

Detection of virus in blood. Virus was detected qualitatively as described elsewhere.[11] Death of mice was recorded during 14 days after inoculation and LD_{50} calculated.[9]

* Presented in part before the 58th General Meeting, Society of American Bacteriologists Chicago, May, 1958.

† Present address: Department of Bacteriology and Immunology, University of Minnesota, Minneapolis (TI) and Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D. C. (JMcC).

* Pigs resident in Hokkaido are infrequently infected with JE virus;[1, 8] therefore shoats farrowed there are less likely to possess maternal antibody than those born near Tokyo.



FIGS. 1–3 *Habitats of pigs on farms.* The farm that contributed 51 pigs to the study in 1956 (Fig. 1) was in a cluster of trees, adjacent to rice paddies, and pigs were kept in stalls within unscreened sheds (Fig. 2). Pigs at other farms were also usually housed in unscreened sheds (Fig. 3 to the left of the house).

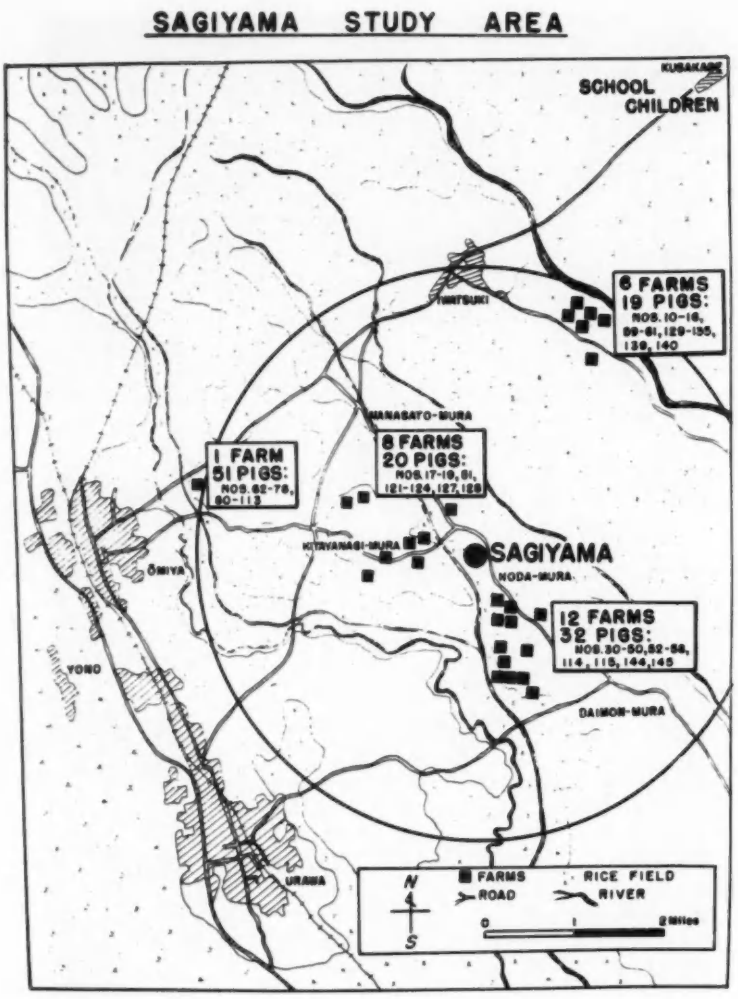


FIG. 4

RESULTS

The frequency and time of occurrence of natural swine infection by JE virus near Tokyo was determined during 1956 and 1957 by study: a) of a 10 to 20% sample of the 500 to 1,000 shoats raised commercially on farms (farm-pigs) near the Sagiyama heronry; and b) of pigs in mosquito traps (trap-pigs) at Sagiyama, Tokyo, Zama and Shinhama. Hemagglutination-inhibition, N, and CF antibody responses to JE virus infection were measured for all pigs; in addition, trap-pigs were observed for signs of illness and in 1956 were tested for viremia.

Frequency and time of infection of farm-pigs near Sagiyama in 1956. Plasmas from shoats on farms near Sagiyama were tested serially for antibodies to JE virus between May and September, 1956. Of 122 pigs, 2 to 10 months of age, 98 were bled in May, and/or in July, and again in September after dissemination of JE virus. In May, 1956, maternal antibody was found in 68 of 80 pigs (85%) by N test (55 with LNI greater than 1.7

TABLE 1

Occurrence of antibodies to JE virus in 98 pigs on farms near Sagiyama, 1956

Antibody		No. of pigs with indicated result/no. tested*		
Tests	Results	21–24 May	24–26 July	3–6 Sept.
Neutralization	LNI > 1.7	55/80 (.69)	13/85 (.15)	97/98 (.99)
	LNI 1.0–1.7	13/80 (.16)	16/85 (.19)	
	LNI < 1.0	12/80 (.15)	56/85 (.66)	1/98 (.01)
Hemagglutination-inhibition	Inhibition at plasma dil. ≧ 1:10	12/80 (.15)	0/95	97/98 (.99)
Complement fixation	Fixation at plasma dil. ≧ 1:4	0/73	0/79	14/89 (.16)

* Each pig was bled in May, and/or in July and in September.

and 13 with LNI 1.0–1.7), in 12 of 80 pigs (15%) by HI test, but in none of 73 pigs by CF test (Table 1). Since in late July, only 34% had detectable N antibody and no pig had detectable HI or CF antibodies, at least two-thirds of the swine were susceptible by early August when widespread virus dissemination began at Sagiyama.[2]

Between late July and early September, 97 of 98 pigs (99%) developed HI antibody to a plasma dilution of 1:20 or greater (Table 1). Concurrently, 96 of 98 pigs (98%) apparently developed N antibody, but only 16% of the 89 pigs with plasmas in sufficient volume for CF test developed CF antibody (Table 1). Actually, in September 97 pigs *possessed* N antibody (Table 1) but only 96 had *developed* it, since one pig retained passively-transferred, maternal, N antibody during August. This pig failed to develop HI antibody by 3–6 September and evidently was protected by maternal antibody from infection by JE virus. Another pig failed to develop N antibody by September despite the development of HI antibody to a plasma dilution of 1:80. This pig was probably infected late in August, too short a time before the September bleeding to develop detectable N antibody. A similar difference between times of appearance of HI and N antibodies has been recorded elsewhere for swine in mosquito traps.[11] The failure of farm-pigs to develop CF antibody in this study is explained by the fact that swine do not uniformly develop antibody detectable by the CF test as employed.[11]

Frequency and time of infection of trap-pigs at Sagiyama, Zama and Tokyo in 1956. Pigs in mosquito traps were tested for appearance of antibody and occurrence of viremia. Like farm-pigs, those in traps had maternal N antibody in May 1956, and six had maternal HI antibody.[11] By early June, antibody was undetectable by HI test, and by 6–9 August, N antibody had disappeared from the plasmas of at least seven of the nine pigs.

Infection by JE virus occurred at Sagiyama and Zama between early July and early August as evidenced by HI antibody development in four pigs (Table 2, pigs 1, 5, 2 and 8). Three pigs were then replaced by three susceptibles in an effort to prevent mosquitoes in traps from ingesting blood containing N antibody to JE virus. This procedure failed to serve its purpose however, since the new pigs (nos. 6, 9 and 4, Table 2) promptly became infected within 8 days of being placed in traps. At Tokyo, trap-pigs did not develop HI antibody until 15–29 August (Table 2), several weeks after the onset of swine infection in the rural areas at Sagiyama and Zama.

Scheduled attempts throughout the season to demonstrate natural viremia in swine were not feasible in 1956 because essentially all personnel and mice were being utilized for mosquito and bird studies. However, in early August when HI antibody was first detected in 4 trap-pigs, blood from the remaining susceptible trap-pigs was inoculated into suckling mice at weekly intervals. JE virus was recovered from two pigs at Sagiyama (nos. 6 and 9 on 21 Aug.) and from one pig in Tokyo (no. 3 on 29 Aug.) (Table 2). Blood from each pig produced signs of encephalitis in 30 to 100% of 1 to 4-day-old mice and the isolated strains of virus were identified by CF test in the second (Sagiyama no. 6 and Tokyo no. 3 strains) or third (Sagiyama no. 9 strain) mouse passages. JE virus was reisolated twice from the August plasma of pig 9 (October 1956 and January 1957) to confirm the presence of viremia.

The sequence of events with pigs 6 and 9 suggested that natural infection of swine by mosquito bite could result in viremia after as short an

TABLE 2

JE virus infection of pigs in Magoon mosquito traps, 1956*

Location	Pig no.	HI antibody titers and occurrence of virus in plasma, by 7-day periods beginning												
		June			July				August					
		15	22	29	6	13	20	27	3	10	17	24	31	
Sagiyama	1	Trapping			<10†				320‡					
	5	began 28 June			<10				640‡					
	6	At lab until 18 August									<10 §	5120‡		
	9										<10	2560‡		
Zama	2	Trapping			<10				320					
	8	began 8 July			<10				80					
	4	At lab until 19 August									<10	80‡		
Tokyo	7	Trapping			<10				<10	<10		40‡	1280‡	
	3	began 14 June			<10				<10	<10		10	640‡	

* Traps were open for entry of mosquitoes three nights per week.

† Numbers are reciprocals of JE HI antibody titers in plasma. All pigs were bled and tested weekly; complete results of HI, N and CF tests are reported elsewhere.[11]

‡ Indicates that no JE virus was recovered upon inoculation of blood into suckling mice.

§ [ ] signifies that JE virus was recovered upon inoculation of blood into mice (21 Aug. for pigs 6 & 9; 29 Aug. for pig 3).

incubation period as 1 to 2 days. Pigs 6 and 9 were moved from the laboratory to the trap on 18 August. However, the trap was opened for ingress of mosquitoes only from 8 p.m. on 19 August to 7 a.m. on 20 August and viremia was found on the next day, 21 August. Five days later HI antibody was present to a high titer and further attempts to recover virus failed on 26 and 28 August (Table 2).

These data from trap-pigs thus paralleled the findings with farm-pigs near Sagiyama but in addition: a) defined more precisely the time of initial swine infection near Sagiyama as late July; b) established that pigs become infected by JE virus in metropolitan Tokyo and in another rural area at Zama nearly 40 miles south of Sagiyama; c) timed swine infection in Tokyo as beginning 2 to 3 weeks later in August 1956 than in the rural areas near Sagiyama and Zama; and d) demonstrated that swine develop viremia following natural infection by JE virus.

However, because relatively small numbers of pigs (12 to 56) were susceptible to JE virus infection before August 1956, it was conceivable that swine infection could have been occurring *in low incidence* during the spring and early summer although undetected. In fact, infection of a few pigs might have been expected in late June or early July, 1956, because infected mosquitoes were present at Sagiyama by then,[3] and were biting pigs in large numbers.[12] To explore this possibility further, an investigation was carried out in 1957 to learn whether pigs became infected by JE virus during the spring and early summer.

Frequency and time of swine infection at Sagiyama and Shinhama in 1957. Beginning in April, 1957, susceptible pigs (without maternal N antibody) were followed for development of HI antibody. Near Sagiyama, pigs were on farms and in traps, but at Shinhama, they were only in traps since there were no pig farms nearby. The farm-pigs near Sagiyama were obtained from two sources, 1) farms in the Sagiyama area, and 2) farms in Hokkaido, the northern island of Japan. Fortunately, since only susceptibles were usable for this study, the pigs from the Sagiyama area in April 1957 had maternal antibodies in lower incidence than found the previous spring (Table 1), *i.e.*, 25/92 (.27) with LNI >1.7, 24/92 (.26) with LNI 1.0–1.7, 43/92 (.47) with LNI <1.0 and 0/92 with HI ≧ 1:10. By mid-May, 1957, 75 susceptibles without detectable N anti-

TABLE 3

JE virus infection of pigs near Sagiyama and Shinhama, 1957

Location	No. of pigs developing HI antibody in plasma/no. susceptible*, by dates of bleeding				
	1 April–23 April	24 April–14 May	15 May–26 June	27 June–28 July	29 July–5 Sept
Sagiyama farms	0/43†	0/76†	0/109	0/54	**33**/34 (.97)
Sagiyama mosquito traps	0/5	0/12	0/12	0/12	**12**/12 (1.00)
Shinhama mosquito traps	0/3	0/3	0/3	**1**/3 (.33)	**3**/3 (1.00)
Total	0/51	0/91	0/124	**1**/69 (.01)	**48**/49 (.98)

* Susceptible = without detectable N antibody (LNI < 1.0).

† Pigs were tested for N rather than HI antibody during these intervals, but were negative for HI antibody in early April and late June.

body, were available from Sagiyama farms. The Hokkaido shoats were obtained in early April to insure an adequate supply of susceptibles since swine from Hokkaido rarely have N antibody to JE virus.[1,3] On 24–25 April only six of 34 pigs from Hokkaido had LNI of 1.0–1.7 and none had detectable HI antibody; upon retest in mid-May, the six equivocals had become negative. Thus, by mid-May 1957 there were 109 susceptible farm-pigs near Sagiyama, 12 trap-pigs at Sagiyama, and three trap-pigs at Shinhama.

No swine infection occurred at Sagiyama and Shinhama: a) during 1 to 23 April, 1957, in 51 susceptible pigs; b) during 24 April to 14 May in 91 pigs; or c) during 15 May to 26 June in 124 pigs (Table 3). Between 27 June and 28 July, none of 66 pigs near Sagiyama and only one of three pigs in the three Shinhama mosquito traps, became infected. This mosquito trap at Shinhama concurrently yielded a strain of JE virus from mosquitoes. However, during 29 July to 5 September, 48 of 49 pigs developed HI antibody simultaneously with an increase in incidence of infected mosquitoes at Sagiyama and Shinhama.[3] Thus, in 1957 there was no evidence of swine infection during the spring and early summer despite the presence of a sizeable vector mosquito population,[3] and the studies from 1956 and 1957 together established that swine infection near Tokyo occurred *after* the appearance of virus in mosquitoes[3] and concurrently with avian infection.[4]

DISCUSSION

Four observations from this and other studies suggest that pigs near Tokyo can serve as sources of JE virus for the vector mosquito, *Culex tritaeniorhynchus*. 1. Under natural conditions swine are frequently infected and develop viremia. 2. Swine viremia lasts long enough (2 to 4 days)[11] for mosquitoes to become infected.[5] 3. Laboratory reared *C. tritaeniorhynchus* transmit virus from pig to pig.[5] 4. *C. tritaeniorhynchus* bite pigs in large numbers in nature.[12]

However, to be sure that swine are *significant* natural sources of JE virus for vector mosquitoes near Tokyo, it remains to show that *large numbers* of *susceptible* pigs exist *each year during the encephalitis season*. To establish this point data on swine population dynamics were obtained from a representative rural prefecture or state (Saitama) near Tokyo. This prefecture contains approximately 1,500 square miles and extends 50 miles from Sagiyama at its farthest point. Between

TABLE 4

*Number of pigs born yearly in Saitama Prefecture, Japan, as recorded from registered matings**

Year	No. of pigs
1950	72,828
1951	74,364
1952	60,206
1953	61,457
1954	59,735
1955	57,463

* Chikusan Jiho, no. 24, 1 Aug. 55 and no. 36, 1 Aug. 56 (Publication of Saitama Livestock Association). These figures represent actual numbers of young pigs registered by owners of breeding male pigs.

TABLE 5

*Numbers of livestock slaughtered yearly in Saitama Prefecture, and throughout Japan, 1948–1955**

Location and Animal	Numbers slaughtered by year							
	1948	1949	1950	1951	1952	1953	1954	1955
Saitama Pref.								
Pig	26,767	68,532	109,563	99,785	157,229	161,438	140,923	134,161
Cow	8,688	7,533	14,354	11,754	9,593	8,271	8,602	13,377
Calf	323	692	2,851	2,532	2,345	2,911	3,739	7,674
Horse	4,449	3,395	3,681	7,001	5,049	5,580	6,356	5,151
Goat	412	85	89	1,964	2,227	2,616	7,013	8,670
Sheep	7	32	169	195	578	975	1,580	1,470
Japan								
Pig	237,707	650,094	1,131,997	922,227	1,525,984	1,843,175	1,432,608	1,659,076
Cow	232,699	237,543	428,375	377,807	374,840	411,538	435,514	601,868
Calf	9,978	15,920	40,890	38,549	41,798	53,447	79,624	208,773
Horse	63,840	58,626	79,904	100,755	87,406	124,025	140,002	123,831
Goat	4,019	2,781	1,852	25,997	31,080	34,000	44,451	41,867
Sheep	269	384	2,295	3,857	8,883	17,650	23,631	23,385

* Statistical Yearbooks of Japanese Ministry of Agriculture and Forestry for years, 1948–1955, published in Dec. 1950, July 1951, March 1952, Oct. 1952, Nov. 1953, Sept. 1954, Nov. 1955 and Nov. 1956, respectively.

TABLE 6

*Age distribution of pigs slaughtered in 1955 at Shibaura Abattoir, Tokyo, Japan**

Age in months	No. of pigs slaughtered	Percentage of pigs slaughtered
0–10	21746	7
10–16	275441	92
16–24	2445	1
	Total = 299,632	

* Annual Report of Shibaura Abattoir, 1955 and personal communication from abattoir authorities in Dec. 1956. In 1955 this abattoir killed 18% of all pigs slaughtered that year in Japan; during 1950–1955, this percentage varied between 18 and 22%.

TABLE 7

*Monthly distribution of pig births in Saitama Prefecture, Japan**

Month of birth	Percentage of pigs born each year
January	6
February	12
March	24
April	12
May	6
June	0
July	0
August	4
September	6
October	20
November	6
December	4

(48, 60; 32, 40)

* Estimates of Dr. Takashi Okada, Veterinarian, and Staff, Veterinary Section, Department of Agriculture & Forestry, Saitama Prefectural Government.

1950 and 1955, 57,000 to 72,000 pigs were born yearly in Saitama (Table 4). Out of such a number in 1956, 98 were studied (roughly a 0.1% sample). Most pigs were raised locally; those moved to other areas for rearing were replaced by pigs brought in for ultimate slaughter, since the numbers of pigs killed yearly were greater than the numbers born (Tables 4 and 5). Pigs immune from past infection were few in number and limited mostly to breeding sows, since individual pigs usually live through only one JE virus season before being slaughtered at 10 to 16 months of age (Table 6). Passive immunity from maternal N antibody does not significantly reduce the numbers of susceptible shoats during the encephalitis season, simply because most pigs are born either

during the months of February–April or during September–November (Table 7) and, by August of each year, are 4 or more months of age and without maternal antibody.* Therefore, in summary, susceptible swine are present each year in large numbers during the encephalitis season near Tokyo and this fact plus those mentioned in the preceding paragraph establish pigs as a major natural source of JE virus for mosquitoes near Tokyo.

However, certain reservations should be placed on extrapolation of these data to the rest of Japan. Certainly times of swine infection are probably different in various parts of Japan, since even between Tokyo City and nearby Saitama prefecture there was a difference of 2 to 3 weeks in 1956. Moreover, much of Japan is mountainous and unlike the Kanto region, and areas such as Hokkaido to the north have low vector-mosquito populations.[7] However, the Kanto area represents well the inhabited coastal regions of Japan[10] and the pigs of Saitama prefecture were a fairly adequate sample (10%) of all swine in Japan (Table 5). Therefore, the possibility certainly has to be considered that wherever *Culex tritaeniorhynchus* and swine coexist in Japan, pigs could serve as a source of virus for mosquitoes. Indeed, even in other parts of the world such as Singapore, there is evidence that pigs may act as a source of JE virus for *C. tritaeniorhynchus*.[6]

The role of domestic animals other than swine as important potential sources of virus for mosquitoes was also investigated in 1956. However, upon inspection of the numbers of cows, horses, goats and sheep slaughtered yearly (Table 5), it became evident that pigs were the most numerous domestic animal. In fact, more pigs were killed yearly between 1949 and 1955 than the combined total of slaughtered cows, calves, horses, goats and sheep. Moreover, these animals were usually killed after 2 or more years of life and thus had more opportunity than swine to develop antibodies and immunity to JE virus.

Finally, it should be emphasized that infection of swine, like avian infection, followed closely in time the onset of detectable mosquito infection,[3] yet preceded human infection by several weeks.[13] The significance of this correlation is discussed elsewhere.[2]

* Farmers limit breeding of sows to February–April and September–November to avoid stillbirths and the financial loss that occurs from JE virus infection of pregnant sows during the encephalitis season (August and early September).

SUMMARY

In 1956 and 1957, 98 to 100% of pigs on farms near Sagiyama north of Tokyo and in mosquito traps at Sagiyama, Tokyo, Zama and Shinhama became infected by JE virus during August. In 1957 extensive studies failed to detect swine infection between April and August. In two rural areas in 1956, swine became infected 2 to 3 weeks before those in Tokyo. Pigs were identified as a major natural source of JE virus for the vector mosquito, *Culex tritaeniorhynchus*, near Tokyo on the basis of: a) a high incidence of natural swine infection; b) the occurrence of viremia following natural infection; c) demonstration in the laboratory that viremia lasts up to 4 days[11] and occurs in titers adequate to infect colonized *C. tritaeniorhynchus* and cause them to transmit virus to pigs and herons;[5] d) the striking propensity of *C. tritaeniorhynchus* to bite pigs in nature;[12] and e) the large, yearly-replenished populations of susceptible pigs near Tokyo.

REFERENCES

1. Bawell, M. B., Deuel, R. E., Matumoto, M., and Sabin, A. B., 1950. Status and significance of inapparent infection with virus of Japanese B encephalitis in Japan in 1946. *Am. J. Hyg.*, **51**: 1–12.
2. Buescher, E. L., and Scherer, W. F., 1959. Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemiologic correlations and conclusions. *Am. J. Trop. Med. & Hyg.*, **8**: 719–722.
3. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., Gresser, I., Hardy, J. L., and Bullock, H. E., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. II. Mosquito infection. *Am. J. Trop. Med. & Hyg.*, **8**: 651–664.
4. Buescher, E. L., Scherer, W. F., McClure, H. E., Moyer, J. T., Rosenberg, M. Z., Yoshii, M., and Okada, Y., 1959b. Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. *Am. J. Trop. Med. & Hyg.*, **8**: 678–688.
5. Gresser, I., Hardy, J. L., Hu, S. M. K., and Scherer, W. F., 1958. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles, from infected pigs and chicks to susceptible pigs and birds. *Am. J. Trop. Med. and Hyg.*, **7**: 365–373.
6. Hale, J. H., Lim, K. A., and Colless, D. H., 1957. Investigation of domestic pigs as a potential reservoir of Japanese B encephalitis virus on Singapore Island. *Ann. Trop. Med. and Parasitol.*, **51**: 374–379.
7. Lacasse, W. J., and Yamaguti, S., 1950. Mosquito fauna of Japan and Korea. Surg. 8th U. S. Army, Japan, p. 235.

8. MITAMURA, T., KITAOKA, M., AND MIURA, T., 1950. On the geographical distribution of Japanese B encephalitis in Far East Asia. *Jap. Med. J.*, **3**: 257–264.
9. REED, L. J., AND MUENCH, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**: 493–497.
10. SCHERER, W. F., AND BUESCHER, E. L., 1959. Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. *Am. J. Trop. Med. & Hyg.*, **8**: 644–650.
11. SCHERER, W. F., MOYER, J. T., AND IZUMI, T., 1959a. Immunologic studies of Japanese encephalitis virus infection in Japan. V. Maternal antibodies, antibody responses and viremia following infection of swine. *J. Immunol.*, **83**: 620–626.
12. SCHERER, W. F., BUESCHER, E. L., FLEMINGS, M. B., NOGUCHI, A., AND SCANLON, J., 1959b. Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors. Zootropism and vertical flight of *Culex tritaeniorhynchus* with observations on variations in collections from animal-baited traps in different habitats. *Am. J. Trop. Med. & Hyg.*, **8**: 665–677.
13. SCHERER, W. F., KITAOKA, M., OKUNO, T., and OGATO, T., 1959c. Ecologic studies of Japanese encephalitis virus in Japan. VII. Human infection. *Am. J. Trop. Med. & Hyg.*, **8**: 707–715.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN

VII. Human Infection*

W. F. SCHERER, M. KITAOKA†, T. OKUNO† AND T. OGATA†

Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan, and the National Institute of Health, Tokyo, Japan

INTRODUCTION

The need to relate human disease to infection of mosquitoes, birds and pigs was obvious throughout these studies on the ecology of Japanese encephalitis (JE) virus. Although vector mosquitoes bite man less frequently than pigs and birds,[8] and humans are infected at lower rates than animals,[1] man, with a longer life span, is eventually infected in high incidence and often enough for encephalitis epidemics to occur. To date, the yearly frequency of human encephalitis has been the only conveniently measured index of virus dissemination to humans. Yet this index inadequately reflects total human infection rates.

Hence, an approach was sought other than attempts to relate size and duration of overt encephalitis epidemics to mosquito, bird and pig infection. Careful consideration suggested that concurrent studies of each host in the same geographic area would be profitable, since frequencies and times of infection could then be correlated among hosts. This type of study was not performed during 1952–1955 because susceptible humans agreeable to frequent and repeated bleedings for antibody determinations were hard to find. The closest approach was made during June–December 1954 by Southam[11] when he found serologic evidence of JE virus infection in five of 30 susceptible children (17%) residing near the Blue Magpie study area at Irumagun (Fig. 2, Article I). Unfortunately, this study did not time infection between June and December, and cannot be confidently related ecologically to infection of mosquitoes, birds and mammals since all hosts were not studied concurrently in the same area.

In 1956 a joint effort of the 406th Medical General Laboratory and the National Institutes of Health in Tokyo made possible measurements of incidence and time of human JE virus infection near Shinhama and Sagiyama where mosquitoes, birds and pigs were concurrently under study. Public support for these investigations and blood samples from children were obtained by members of the Japanese N.I.H. Equipment, technical assistants for bleeding, and facilities for serologic testing were provided by the 406th MGL.

This articles describes: a) the frequency and time of human infection by JE virus during 1956 near the Shinhama and Sagiyama study sites for mosquitoes, birds and pigs; and b) the yearly, summer epidemics of overt human JE near Tokyo. Correlation of these data with infection of mosquitoes, birds and pigs will be presented in the final article of this series.

MATERIALS AND METHODS

Humans near Shinhama and Sagiyama. Four hundred and four children were observed during June–November 1956 for significant increases in plasma concentrations of JE virus antibodies and for manifestations of clinically apparent encephalitis. Two hundred and four children, ages 6 to 12, were at the Minami-gyotoku primary school, 1 to 2 miles across rice paddies and lotus fields from the Shinhama heronry (Fig. 2, Article I); their dates of bleeding were 15 June, 16 July, 17 August, 14 September and 29 October, 1956. Another 200 children, ages 6 to 8, were studied at a school in Kasukabe 5 miles from Sagiyama (Fig. 2, Article I) across intervening rice paddies and upland dry farms. These children were bled on 19 June, 19 July, 21 August, 19 September and 2 November 1956. Children were younger at Kasukabe than at Minami-gyotoku because the schoolmaster permitted us to select the youngest children to obtain the largest numbers of susceptibles, *i.e.*, those without detectable neutralizing (N) antibody.

Heparinized blood was obtained and N, hemagglutination-inhibition (HI) and comple-

* Presented in part before the 32nd Annual Meeting, American Association of Immunologists, April, 1958.

† Present address: The National Institute of Health, Takanawa, Tokyo (M. K., T. O., and T. O.).

ment fixation (CF) antibodies determined on plasma as reported elsewhere.[2] For detection of JE virus in blood, mice, 1 to 4 days of age, from one litter, were inoculated with heparinized whole blood, 0.01 ml intracranially and 0.02–0.03 ml subcutaneously immediately upon bleeding. Upon return that day to the 406th MGL, mice were observed daily for 14 days and handled as described previously.[3, 4]

Human encephalitis in Tokyo-to. Tokyo-to or county includes the city (shaded area between Tama and Ara rivers, Fig. 2, Article I), a less densely populated, more rural region to the west, and several nearby Izu islands; its human population in 1956 was approximately 8 to 9 million. The Municipal Health Department recorded yearly each case of clinically apparent encephalitis in Tokyo-to, but since most cases were not confirmed serologically, the reported cases undoubtedly include diseases other than JE.

RESULTS

In 1956 frequencies and times of human infection by JE virus were determined in children near Shinhama and Sagiyama for correlation with similar data concerning infection of mosquitoes, birds and pigs. Graphs are presented of clinically apparent human encephalitis in Tokyo-to for crude yearly comparison with other ecologic observations during 1952–1956.

Inapparent Human Infection by JE Virus Near Shinhama and Sagiyama

Antibody tests were used to determine infection since, in birds, viremia tests had been shown to be less sensitive.[4] Children 6 to 12 years of age were chosen because preschool children would have been less cooperative and impossible to bleed in the centralized, efficient manner employed at schools, and because older age groups would have provided fewer susceptibles.[1] Preseasonal, seasonal and postseasonal plasmas were obtained from 204 of 226 children near Shinhama and from 200 of 280 children near Sagiyama (Table 1). Preseasonal plasma from each child (June or July) was tested for N antibody.

Frequency of infection prior to June 1956. Based on previous serologic surveys,[1] the N test was used to detect past JE virus infection and to identify susceptible children.[5] Tests of preseasonal plasmas revealed that approximately one-fourth of the children in each school possessed detectable N antibody to JE virus (Table 2). Of interest were the higher incidences of N antibody in children 6 to 8 years of age at Sagiyama (.26–.28) than at Shinhama (.08–.16). This finding suggested that during the 6 to 8 years prior to 1956, children near Sagiyama had been more frequently infected by JE virus than children near

TABLE 1

Procurement of serial plasmas from children near Shinhama and Sagiyama, June–November, 1956

Bleeding schedule	Time of procurement of plasma			Number of children	
	Preseason	Season	Post-season	Shinhama (Minami-gyotoku)	Sagi-yama (Kasu-kabe)
Complete	June and/or July	Aug and/or Sept	Oct or Nov	204	200
Incomplete	June and/or July	Aug and/or Sept	None	9	10
	June and/or July	Aug; no Sept	None	13	70
			Total	226	280

TABLE 2

Incidence of JE virus N antibody in children near Shinhama and Sagiyama, June, 1956

Age of children in years	Number of children with N antibody in plasma*	
	Shinhama (Minami-gyotoku)	Sagiyama (Kasukabe)
6	1/12 (.08)†	10/36 (.28)
7	3/35 (.09)	40/149 (.27)
8	6/37 (.16)	15/57 (.26)
9	8/39 (.21)	
10	10/36 (.28)	
11	14/33 (.42)	
12	13/28 (.46)	
Totals.....	55/220 (.25)	65/242 (.27)

* Only two children at Shinhama and six at Sagiyama had LNI = 1.0–1.7; all others were greater than 1.7.

† Numerator signifies number of children with N antibody in plasma; denominator indicates number of children tested; ( ) represents decimal fraction with antibody.

TABLE 3

Patterns of antibody to JE virus in children near Shinhama and Sagiyama, June–November, 1956

Antibody patterns		Numbers of children by site and age in years*											
		Shinhama (Minami-gyotoku)								Sagiyama (Kasukabe)			
N antibody in June or July	HI antibody in Oct or Nov	6	7	8	9	10	11	12	Total	6	7	8	Total
I. Absent†	Absent†	9	30	27	29	23	16	12	146	20	75	40	135
II. Absent†	Present†	1	0	2	0	2	0	2	7 (7/153 = .05)	2	4	1	7 (7/142 = .05)
III. Present†	No change in titer greater than two-fold‡	1	2	6	7	7	12	12	47	8	32	15	55
IV. Present†	Rise in titer ≧ fourfold	0	0	0	1	1	2	0	4 (4/51 = .08)	1	2	0	3 (3/58 = .05)

* Of 19 additional children bled serially only through September 1956, seven followed pattern I, and two, pattern III at Minami-gyotoku; at Kasukabe, eight fitted pattern I, and two, pattern III.

† N antibody absent = LNI < 1.0; present = LNI > 0.9. HI antibody absent = titer < 1:10; present = titer ≧ 1:10

‡ HI antibody titers ranged from 1:10 to 1:40.

Shinhama. Also noteworthy is the failure of infection frequencies to increase for ages 6, 7 and 8 at Sagiyama. The expected pattern is for N antibody frequencies to increase with age[1] as at Shinhama (Table 2). Why this did not occur for the three ages studied at Sagiyama is unknown.

Frequency of infection during June–November 1956. Preseasonal plasmas were tested for N and HI antibodies, but seasonal and postseasonal plasmas were initially subjected only to HI test because of its extreme sensitivity for detecting antibody promptly following overt human JE,[3] and because of its inexpensiveness and ease of performance. Only selected seasonal and postseasonal plasmas were tested for CF and N antibodies. Plasmas from children who showed significant rises in antibody levels were retested simultaneously by all three tests.

On the basis of absence or presence of N antibody in preseasonal plasma from June or July 1956, each child was classified as *potentially susceptible* or *possibly immune* to JE virus. These children were then further classified into two additional groups based on their HI antibody response by October–November 1956. Thus, four antibody patterns resulted by which children were grouped: I, *potentially susceptible* and *noninfected* children with JE virus antibody undetectable throughout the entire June–November period; II, *potentially susceptible* and *infected* children who had no antibody in June, but developed it subsequently; III, *possibly immune* and *not reinfected* children who possessed antibody that remained unchanged; and IV, *possibly immune* and *reinfected* children who possessed antibody in June, but showed significant antibody increases between June and November 1956. Since pattern IV was somewhat surprising and suggested the possible existence of another Group B arthropod-borne virus in the Shinhama and Sagiyama study areas, this possibility was carefully evaluated.[7] However, it was considered highly unlikely and thus all four antibody responses are interpreted here as caused by JE virus. The titers of CF, HI and N antibodies are described elsewhere.[8]

At Shinhama seven of 153 (.05) susceptible children developed HI antibody and four of 51 (.08) children with N antibody in June or July showed 4-fold or greater rises in HI antibody (Table 3). At Sagiyama seven of 142 (.05) and three of 58 (.05) children developed antibody or showed significant rises in HI antibody levels. In no case was encephalitis clinically evident[9]. Age of individual children within the range

TABLE 4

Chronology of JE virus infection of children near Shinhama and Sagiyama, June–November, 1956

Location	N antibody in June or July	No. of children by time of development or rise in HI antibody				
		June 15, 19	July 16, 19	Aug 17, 21	Sept 14, 19	Oct, Nov 29, 2
Shinhama	Absent*	0	0	0	1	6
	Present	0	0	0	3	1
	Total . . .				4	7
Sagiyama	Absent	0	0	0	7	0
	Present	0	0	0	3	0
	Total . . .				10	

* See footnote† Table 3.

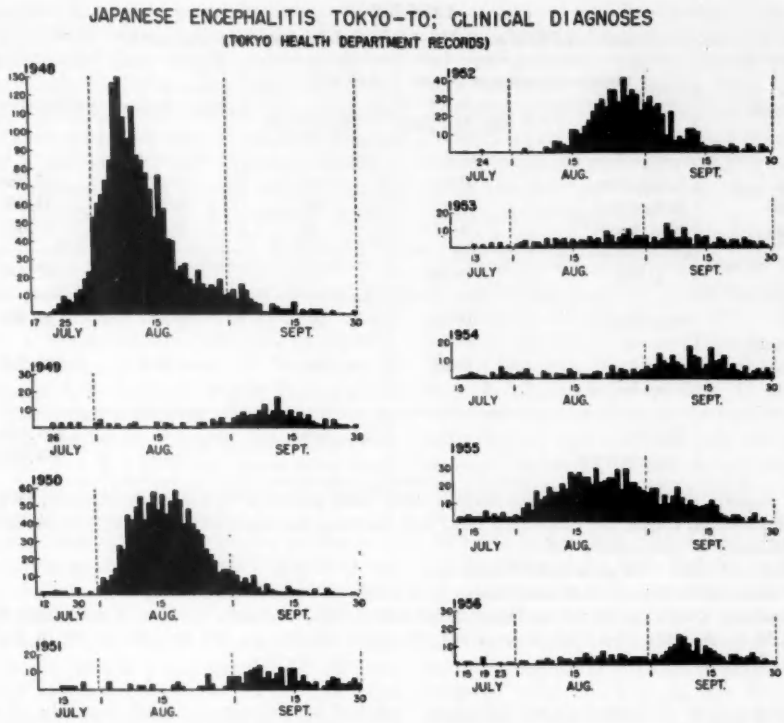
6 to 12 years had no apparent effect on occurrence of infection (Table 3); indeed, from their relationships to environment, one would not expect children between ages 6 and 12 to have significantly different degrees of contact with mosquitoes.

Time of infection during June–November 1956. The chronology of infection by JE virus in children near Shinhama seemed to differ from that near Sagiyama. While all ten antibody responses near Sagiyama occurred between mid-August and mid-September, only four of eleven Shinhama children showed an antibody response by mid-September whereas the other seven responded between mid-September and late October (Table 4). After critical review of these data, the most likely interpretation was that the spread of JE virus ceased among humans near Sagiyama after early September whereas at Shinhama mosquito transmission of virus continued beyond mid-September. Since all seven children with primary antibody responses near Sagiyama manifested them by 19 September and since HI antibody appears in plasma within 5 to 7 days of onset of overt disease,[3] it could be calculated that the Sagiyama infections probably occurred on or before 12–14 September. At Shinhama, on the other hand, six primarily infected children had no HI antibody when bled on 14 September, suggesting either that they had just become infected within the previous few days or that their infections occurred at a later date, subsequent to those at Sagiyama.

Attempts to recover JE virus from blood. On the remote chance that viremia might be detected during August and September 1956, blood from children without N antibody in June or July was inoculated into suckling mice within 1 hour of venipuncture. The number of bloods inoculated by place and date were as follows: Shinhama, 42 on 17 August and 39 on 14 September; Sagiyama, 36 on 21 August and 36 on 19 September. No virus was recovered. In retrospect, only seven of these bloods were from children who showed evidence of JE virus infection by antibody tests and only two children (bled in August at Sagiyama) were tested for viremia at a time close to, but before, their period of infection. These results emphasized that testing of populations of inapparently infected individuals for viremia will be successful only if infection rates are considerably greater than the 5% present in this group, or if hundreds to thousands of individuals are sampled.

Overt Human Japanese Encephalitis in Tokyo-to

The yearly occurrences of clinically apparent JE in Tokyo and the numbers and time distributions of cases from Japan diagnosed by serologic tests at the 406th MGL are presented in Figure 1. It can be seen that the numbers and times of encephalitic illnesses varied yearly between 1948 and 1956. Of interest was the occurrence of larger epidemics in August and smaller epidemics in September. Large epidemics did not occur in regular cycles although a biyearly cycle was suggested from 1948 through 1953. In support of the thesis that reported encephalitis cases in Tokyo-to actually reflected the occurrence of JE, it should be noted that yearly numbers of serologically proved cases from Japan (406th MGL) were roughly proportional to cases in Tokyo (Fig. 1). Moreover, the times of occurrence of serologically proved cases and the recoveries of JE virus from brains of fatal cases corresponded to the encephalitis epidemics. Strains of JE virus were isolated from patients whose onsets of illness were 4 and 8 August, 1948, 16 and 30 September 1949, 13 and 16 August 1950, 1 August and 5 September 1955, and 10 September 1956. These crude correlations of clinically and serologically diagnosed illnesses thus suggest that JE virus was the major etiologic agent causing the yearly fluctuations in encephalitis near Tokyo between 1948–1956.



YEAR		1946	1947	1948	1949	1950	1951	1952	1953	1954	1955	1956[a]
TOKYO AREA	CASES	0	1	1943	215	1182	181	711	231	585	761	285
	DEATHS	0	1	524	70	292	45	178	61	110	218	95
JAPAN	CASES	201	253	4757	1284	5196	2188	3545	1729	1733	3686	3988
	DEATHS	99	228	2820	1177	2430	996	1433	719	655	1502	1499
	LABORATORY DIAGNOSES ADS INCL.			10 (2-26 AUG)	10 (19 AUG-30 SEP)	12 (7 AUG-21 SEP)	1 (ONSET NOT GIVEN)	12 (9 AUG-24 OCT)	2 (24 AUG-4 SEP)	6 (? AUG-? SEP)	20 (1 AUG-16 SEP)	6 (17 AUG-10 SEP)

a TOKYO 1 JAN-31 DEC 56; JAPAN 1 JAN-20 OCT 56.

FIG. 1

DISCUSSION

During 1948–1956 human infection by Japanese encephalitis virus was manifested near Tokyo by yearly epidemics of central nervous system disease. Epidemics varied in size and in time of occurrence during the summer, although no consistently recurrent cycle was evident. Large epidemics occurred mainly in August whereas small ones were chiefly in September. For comparison with observations of mosquitoes and birds at the Shinhama and Sagiyama heronries near Tokyo, the data concerning epidemics in Tokyo-to were of limited usefulness since: a) they pertained to a metropolitan area of different ecology and at least quantitatively different mosquito fauna than Shinhama or Sagiyama; b) the records were based on clinical diagnoses usually not confirmed by laboratory tests and therefore included encephalitis from infectious agents other than JE virus; and c) they provided a measure only of JE virus *disease* in man and did not necessarily reflect frequencies of inapparent or total human *infection*. Obviously, virulence differences among virus strains might cause fluctuations in yearly disease rates despite

TABLE 5

*Populations of children near Shinhama and Sagiyama in May, 1956**

Study sites	Districts surrounding or adjacent to study sites†			Ages of children	Numbers of children
	Name	Miles from study site	Approx. size in sq. mi.‡		
Shinhama (Minami-gyotoku)	Ichikawa	0–5	20	6–12	17,784
	Edogawa	1–4	18	6–12	34,558
	Funabashi	3–10	32	6–12	15,539
	Total	0–10	70		67,881
Sagiyama (Kasukabe)	Saikatsu	0–15	160	6–8	28,518
	Saitama prefecture	0–50	1523	6–8	188,255
Tokyo	Tokyo prefecture	—§	818	6–8 6–12	574,659 980,086

* From Reports on Prefectural Public Schools, May 1956, prepared by Research Sections, Departments of Education, Chiba (for Shinhama area) and Saitama (for Sagiyama area) or from Statistical Section, Ministry of Education, Tokyo.

† See Fig. 2, Article I for geographic locations.

‡ From Geographic Survey Institute, Ministry of Construction, Tokyo.

§ The limits of Tokyo prefecture on Honshu, the main island of Japan, are 1 to 50 miles from Shinhama and 10 to 45 miles from Sagiyama. A few thousand children on the Izu islands are in Tokyo, but not on Honshu.

little accompanying variation in total infection rates.

Therefore, in 1956 experiments were carried out to measure human infection by JE virus concurrently with studies of mosquitoes, birds and pigs. These experiments disclosed a human infection frequency of 5%. The times of infection were mid-August to the end of October at the Shinhama study site and mid-August to mid-September near Sagiyama. The absence of human infection after mid-September in the children studied near Sagiyama was of unknown significance, but certainly did not result from exhaustion of the supply of susceptibles or from a lack of infected mosquitoes during September.[3] The fact that the infection frequencies near Shinhama were equal to those at Sagiyama was also of interest, although it is noteworthy that the frequencies near Shinhama and Sagiyama evidently were not comparable during the 6 to 8 years prior to 1956 (Table 2). The similarity in infection frequencies in 1956 may have occurred by chance, or because the areas were ecologically similar and contained mosquitoes and birds infected by JE virus.[3, 4] Unfortunately, it was not feasible to include a control group of children from elsewhere in the Kanto plain to learn whether human infection was less frequent away from the dense mosquito populations present at heronries, than near heronries. Thus, the infection incidences of 5% found in these studies can be applied in the strict sense only to children living in the Shinhama and Sagiyama areas.

Of importance to emphasize, however, is the fact that in Japan a human infection frequency of 5% represents a large number of infected individuals because the total population is so large. To illustrate this point, populations of children in the Shinhama and Sagiyama areas are presented in Table 5. Within 10 miles of the Shinhama school at Minami-gyotoku were the ecologically similar districts of Ichikawa, Edogawa and Funabashi (Fig. 2, Article I), totaling approximately 70 square miles and containing 67,881 children ages 6 to 12 years in May, 1956. Thus, the 204 children studied near Shinhama were roughly a 0.3% sample of the total population. By multiplying the total

population by the infection frequency in the sample, (67,000 × .05), one finds that 3,350 children could have become infected by JE virus during June–November, 1956, near Shinhama. Likewise, in Saikatsu, the district around the Sagiyama school (Fig. 2, Article I), 28,518 children ages 6 to 8 years provided a potential 1,400 children (28,000 × .05) infected by JE virus in 1956. Here the 200 children studied represent approximately a 0.7% sample. Direct extrapolation of these infection incidences to larger geographic regions such as Saitama prefecture or to areas of distinctly different terrain such as Tokyo, however, may not be justified. Yet obviously, if the infection frequencies of Shinhama and Sagiyama could be applied to the more than 1 million children residing in Saitama and Tokyo (Table 5), over 50,000 children in the 6 to 12-year-age group alone could conceivably have become infected by JE virus during June–November 1956.

Also of interest were the similar incidences of recent infection in children with and without pre-existing JE virus N antibody. Prior to this study it was uncertain whether plasma antibody levels in humans with pre-existing antibody would rise during mosquito dissemination of JE virus, though suggestive evidence for this was found in studies of vaccinated humans by Tigertt *et al.*[12] That such antibody responses occurred was particularly intriguing in considerations of immunity and pathogenesis of human JE virus infection.[9] However, in addition, an interesting epidemiologic concept was suggested by this observation. If one accepts that JE virus was the only Group B arthropod-borne virus in the Tokyo area during 1952–1956[7] and thus that HI antibody rises in children with pre-existing N antibody were due to reinfection by JE virus,[9] one can conclude that the HI test will detect recent human infection by JE virus in populations at large, irrespective of their previous experience with the virus. This would mean, from the practical viewpoint, that previously infected humans would be as useful as noninfected individuals for measurement of exposure frequencies to JE virus in Japan. Certainly infected mosquitoes would not selectively bite humans without pre-existing antibody, in preference to those with antibody, and thus one would expect equal exposure frequencies for both groups under similar ecologic conditions. Obviously, further studies are indicated to confirm or refute this implication and to attempt application of this finding to other animals commonly infected by JE virus, *e.g.*, pigs and birds. Unfortunately, these statements probably apply only to northeast Asia because in tropical areas where JE virus coexists with other Group B arthropod-borne viruses, one could not distinguish between heterotypic and homotypic HI antibodies.

Another concept of epidemiologic interest was suggested by the observation that at Shinhama the incidences of previous human infection, as evidenced by N antibody, increased progressively between ages 6 to 12 years (Table 2). Assuming that N antibody in most humans remains detectable in plasma for at least 12 years after infection, and that children by 6 months of age, after loss of maternal antibody, have a zero antibody incidence, the increments in N antibody frequencies for the Shinhama children might be ascribed to certain calendar years, *i.e.*, .08 for 1950–55, .01 for 1949, .07 for 1948, .05 for 1947, .07 for 1946, .14 for 1945, and .04 for 1944. The obvious question then arose whether years with large increments in antibody incidence corresponded to years of epidemic disease. Unfortunately, accurate records of human JE for 1944–47 were not kept in the Shinhama or Sagiyama areas or even in Tokyo-to to extend the data presented in Figure 1 for 1948–1956. Thus, correlations could not be made between epidemics and calculated yearly differences in infection frequencies. However, this approach to analysis of data might be useful in the future when infection incidences are determined for other groups of humans.

In summarizing present knowledge of human infection by JE virus in Japan, it is evident that yearly frequencies of total, apparent and inapparent, new infections are in the 1 to 20% range. In this study near Tokyo the value for 1956 was 5% and for 1944–1949, 1 to 14%. Tigertt *et al.*,[12] found that 11 and 12% of 114 and 128 children at Okayama, Japan inapparently developed N antibody during the July–September intervals of 1948 and 1949, and 15% of 26 children developed CF antibody in 1948. In 1950 he found 1 of 17 children (6%) developing N antibody between June and November. Southam's report[11] of studies at Irumagun near Tokyo revealed 17% of 30 children infected during June–December 1954. Whether these

values apply to other geographic areas, in particular the tropics, remains to be determined.

Also undetermined to date is the role of man in the natural dissemination of JE virus. Whether infected humans are important sources of virus for mosquitoes is difficult to evaluate conclusively because incidence and duration of human viremia are unknown and will probably remain so. Obviously man cannot be inoculated experimentally and tested for viremia and, as mentioned under *Results*, humans with natural viremia will not be found frequently enough for detailed study unless infection rates in future epidemics become considerably greater than the usual 5 to 15%, or unless hundreds to thousands of individuals are tested. However, even if one assumes that man, like birds and pigs, has viremia of several days duration[5, 10] and in sufficient titer to infect *C. tritaeniorhynchus*,[6] it seems likely that man's contribution to the infection of vector mosquitoes near Tokyo is insignificant compared with that of pigs and birds, simply because man is bitten so much less frequently.[8] Thus, from a functional viewpoint of virus ecology, human infections are probably so-called "dead end" infections, though conceivably occasional humans might infect a few mosquitoes.

SUMMARY

Inapparent human infection by JE virus occurred during June-November 1956 in 5% of 404 school children ages 6 to 12 years near the Shinhama and Sagiyama study sites for mosquitoes, birds and pigs. Because human populations were large, this percentage represented a large number of infected children. Infection was independent of the child's age. Children developed antibody during a shorter time interval near Sagiyama (21 August to 19 September) than near Shinhama (17 August to 29 October). Children with pre-existing neutralizing antibody manifested the same JE virus exposure frequency by hemagglutination-inhibition antibody test as children without pre-existing antibody.

Past infection frequencies for JE virus as reflected by neutralizing antibody incidences in June or July 1956 were higher in children 6 to 8 years of age near Sagiyama (.28–.26) than near Shinhama (.08–.16). The percentages of previously infected children cumulated yearly for ages 6 to 12 years at Shinhama, but at Sagiyama past infection frequencies were the same for ages 6, 7 and 8.

Between 1948 and 1956 clinically apparent Japanese encephalitis near Tokyo occurred yearly during August and September. The size of epidemics varied, but correlated roughly with the numbers of serologic diagnoses of JE made yearly at the 406 MGL. No uniform cycle of recurrence was evident, although large epidemics occurred more often in August whereas small epidemics were usually in September.

REFERENCES

1. Bawell, M. B., Deuel, R. E., Matumoto, M., and Sabin, A. B., 1950. Status and significance of inapparent infection with virus of Japanese B encephalitis in Japan in 1946. *Am. J. Hyg.*, **51**: 1–12.
2. Buescher, E. L., Scherer, W. F., Grossberg, S. E., Chanock, R. M., and Philpot, V. B., Jr., 1959a. Immunologic studies of Japanese encephalitis virus in Japan. I. Antibody responses following overt infection of man. *J. Immunol.*, **83**: 582–593.
3. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., Gresser, I., Hardy, J. L., and Bullock, H. R., 1959b. Ecologic studies of Japanese encephalitis virus in Japan. II. Mosquito infection. *Am. J. Trop. Med. & Hyg.*, **8**: 651–664.
4. Buescher, E. L., Scherer, W. F., McClure, H. E., Moyer, J. T., Rosenberg, M. Z., Yoshii, M., and Okada, Y. 1959c. Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. *Am. J. Trop. Med. & Hyg.*, **8**: 678–688.
5. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., and McClure, H. E., 1959d. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses in birds, *J. Immun.*, **83**: 605–613.
6. Gresser, I., Hardy, J. L., Hu, S. M. K., and Scherer, W. F., 1958. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* from infected pigs and chicks to susceptible pigs and birds. *Am. J. Trop. Med. and Hyg.*, **7**: 365–373.
7. Scherer, W. F., and Buescher, E. L., 1959. Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. *Am. J. Trop. Med. & Hyg.*, **8**: 644–650.
8. Scherer, W. F., Buescher, E. L., Flemings, M. B., Noguchi, A., and Scanlon, J., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors. Zootropism and vertical flight of *Culex tritaeniorhynchus* with observations on variations in collections from animal baited traps in different habitats. *Am. J. Trop. Med. & Hyg.* **8**: 665–677.
9. Scherer, W. F., Kitaoka, M., Grossberg, S. E., Okuno, T., Ogata, T., and Chanock, R. M., 1959b. Immunologic studies of Japanese encephalitis virus in Japan. II. Antibody responses following inapparent human infection. *J. Immunol.*, **83**: 594–604.
10. Scherer, W. F., Moyer, J. T., and Izumi, T., 1959c. Immunologic studies of Japanese encephalitis virus in Japan. V. Maternal

antibodies, antibody responses and viremia following infection of swine. *J. Immunol.*, **83:** 620–626.

11. SOUTHAM, C. M., 1956. Serological studies of encephalitis in Japan. II. Inapparent infection by Japanese B encephalitis virus. *J. Infect. Dis.*, **99:** 163–169.

12. TIGERTT, W. D., BERGE, T. O., BURNS, K. F., AND SATTERWHITE, J. P., 1956. Evaluation of Japanese B encephalitis vaccine. IV. Pattern of serologic response to vaccination over a five-year period in an endemic area (Okayama, Japan). *Am. J. Hyg.*, **63:** 238–249.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN.

VIII. Survey for Infection of Wild Rodents

W. F. SCHERER, E. L. BUESCHER, C. M. SOUTHAM*, M. B. FLEMINGS AND A. NOGUCHI

Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

INTRODUCTION

Wild rodents were considered possibly important in the natural history of Japanese encephalitis (JE) virus in Japan because they were susceptible to virus, had large yearly population turnovers, and were prevalent and widely distributed throughout the Kanto plain around Tokyo. Young rodents, produced in abundance each year, could, if bitten by mosquitoes, conceivably become infected and, if viremic for a sufficient time, serve as sources of virus for additional mosquitoes. Although it is unknown whether *Culex tritaeniorhynchus*, the major vector of JE virus in Japan, frequently bites wild rodents,† it was considered desirable to survey rodents for evidence of JE virus infection as an initial step in understanding their place, if any, in the natural history of JE virus. Therefore, during the years 1953–1957, rodent sera or plasmas were tested for JE virus antibodies. The neutralization (N) test was considered to be the most sensitive available measure of past infection by JE virus because N antibody in human and animal sera usually persists for months to years following infection.[2, 3, 7, 9] Contrariwise, the recovery of infectious JE virus from blood would have been an insensitive test for rodent infection because virus exists in blood of most animals for less than a week following infection. The test for hemagglutination-inhibiting (HI) antibody was also used, but since this antibody becomes undetectable in some birds within a year of infection,[3] the HI test was used for rodent plasmas collected only during the summer encephalitis season and the Fall thereafter. In 1953, N antibody was searched for in sera from rodents collected at the Shinhama heronry, and in areas of Tokyo where naturally infected *Culex tritaeniorhynchus* were known to occur.[1] In 1954, HI tests were performed on plasmas obtained in June–July and in November from rats trapped in Tokyo City by the Tokyo Municipal Health Department. From September, 1956 through February, 1957, wild rodents were caught in the rural areas and foothills of the Kanto plain and tested by N and/or HI tests.

MATERIALS AND METHODS

Rodents were trapped alive in wire or wooden traps and bled in Tokyo, either at the 406th Medical General Laboratory or, in 1956–1957, at the 207th Medical Detachment Laboratory. Cardiac puncture was used to obtain serum in 1953 and heparinized plasma in 1954 and 1956. Neutralization tests were done in 1953 with undiluted, unheated serum mixed with approximately 10 and 100, or only 100, weanling-mouse-LD_{50} doses of JE virus. In 1956–1957 unheated plasmas were tested either undiluted or after dilution in saline or in antibody-free serum (guinea pig or rabbit, stored at −20°C) to make sufficient volume to test versus 5, 50 and 500 intracranial weanling-mouse-LD_{50} doses of virus. Strain M1/311 of JE virus was employed in mouse passage 10 or 11 from its natural source, *C. tritaeniorhynchus*. Occasionally plasmas were tested only versus 5 and 500 LD_{50} doses of virus. Other details of the N tests are described elsewhere.[4] HI antibody tests were performed by the standard method[4] with 8 units of hemagglutinin versus 2-fold dilutions of heated (56°C, 30 min.), acetone-extracted plasma starting at 1:10.

RESULTS

Between November, 1953, and January, 1954, 95 rodents were caught at the Shinhama heronry and in areas of Tokyo where naturally infected *C. tritaeniorhynchus* were known to occur.[1] Although the records of rodent species are no longer available, no JE virus N antibody was found in these sera.

During June and July, 1954, plasmas from 87 *Rattus norwegicus* and 11 *R. rattus* trapped in Tokyo failed to show JE HI antibody (Table 1).

* Present address: Sloan-Kettering Institute for Cancer Research, New York (C. M. S.)

† Observations of albino mice in mosquito traps at the Sagiyama heronry in 1956 suggested that *C. tritaeniorhynchus* bites mice infrequently.[6]

TABLE 1

Results of N and HI tests for JE virus antibodies in rodents from urban and rural areas near Tokyo, Japan

Genus and species	Location	Year	Months	No. pos/no. equiv./no. tested by neut*	No. pos./no tested by HI†
Rattus norwegicus	Tokyo City	1954	June, July		0/87
	Tokyo City	1954	Nov.		0/118
Rattus rattus	Tokyo City	1954	June, July		0/11
	Tokyo City	1954	Nov.		0/54
	Kanto Plain	1956–57	Sept.–Feb.	0/0/22	0/2
Microtus montebelli montebelli	Kanto Plain	1956–57	Sept.–Feb.	0/1/3	0/4
Apodemus geisha	Kanto Plain	1956–57	Sept.–Feb.	0/6/47	0/49
Apodemus speciosus	Kanto Plain	1956–57	Sept.–Feb.	1/4/56	1/44
Mus musculus	Kanto Plain	1956–57	Sept.–Feb.	0/0/2	0/2
Utitruchus talpoides hondonis	Kanto Plain	1956–57	Sept.–Feb.	0/1/1	0/1
Sorex caecutiens shinto	Kanto Plain	1956–57	Sept.–Feb.		0/1
Totals				1/12/131	1/373

* Positive = LNI greater than 1.7; equivocal = LNI 1.0 – 1.7

† Positive = inhibition of hemagglutination by a dilution of plasma greater than, or equal to, 1:10.

In November, 1954, although JE virus had disseminated in nature during the interval since July, plasmas from 118 *R. norwegicus* and 54 *R. rattus* were without detectable JE HI antibody. That rats could be expected to form HI antibody within several months following naturally acquired JE virus infection was based on the demonstration of HI antibody development from titers of less than 1:10 to 1:80 or greater in 3 *R. norwegicus*, 10 to 14 days after intracerebral inoculation of JE virus, strain M1/311, as infected-mouse-brain suspension. Moreover, albino mice form HI antibody following inoculation with virus[5] and one *Apodemus speciosus* collected in nature during September 1956 had HI antibody to a titer of 1:80 (Table 1).

From September, 1956, through February, 1957, plasmas were collected from 163 rodents caught in rural areas throughout the Kanto plain and foothills to the north, west and south of Toyko (see Fig. 2, Article I). Of the 163 rodents, 71 were tested by N and HI tests, 60 by N test only, and 32 by HI test only. Of the 131 plasmas tested for N antibody, 29 were tested undiluted, 48 were diluted 1:3 to 10 in saline, 21 were diluted 1:5 to 100 in guinea pig serum and 33 were diluted 1:5 to 60 in rabbit serum to make the volume sufficient for test versus three quantities of virus. The results of antibody tests are given in Table 1.

One *Apodemus speciosus* caught west of Tokyo at Fuchu in September, 1956 had HI antibody in titer of 1:80; N test of this plasma diluted 1:3 in saline showed a log N index (LNI) of 0.0. These results suggested that this animal had been recently infected by JE virus and had not yet developed N antibody. Similar observations have been made in humans, birds and pigs.[3, 7, 8] Another *Apodemus speciosus* collected north of Tokyo at Karuizawa in October, 1956, had N antibody (LNI greater than 3.0) in plasma diluted 1:10 in saline, but no HI antibody at 1:10 dilution. This rodent was probably like the humans who, after an infection in the distant past, retained detectable levels of only N antibody.[4, 7] Twelve plasmas neutralized virus in the equivocal range: 2 at 1.4 LNI, 1 at 1.2 LNI and 9 at 1.0 LNI. Actually, it is possible that the LNI of some of these plasmas may have been in the positive range since they all were diluted with saline, rabbit serum or guinea pig serum from 1:5–1:30 to obtain volume sufficient to test. Two plasmas with LNI of 1.4 were diluted 1:5 and 1:10 in rabbit serum and that with LNI of 1.2 was diluted 1:5 in saline; they were from *Microtus montebelli montebelli*, *Apodemus speciosus* and *Apodemus geisha* and were collected north and west of Tokyo at Urawa, Camp Drake and Fuchu, respectively.

SUMMARY

Neutralization and/or hemagglutination-inhibiting antibody tests of sera and plasmas from rodents collected in urban and rural areas near Tokyo, Japan failed to show evidence of frequent infection of wild rodents by JE virus during 1953–1957. Plasma from only one of 131 rodents tested in 1956 unequivocally neutralized virus and only one of 373 rodents tested by the hemagglutination-inhibition test showed antibody in heated, acetone-extracted plasma. These results strongly suggest that wild rodents play an unimportant role in the ecology of JE virus near Tokyo despite their large yearly population turnover. Perhaps their low infection rate reflects infrequent biting by *Culex tritaeniorhynchus*, the vector of JE virus in Japan.

REFERENCES

1. Barnett, H. C., 1951. Virus isolation studies on mosquito material collected in 1950 in Tokyo. Annual Professional Report, 406 Medical General Laboratory, pp. 231–239.
2. Bawell, M. B., Deuel, R. E., Matumoto, M., and Sabin, A. B., 1950. Status and significance of inapparent infection with virus of Japanese B encephalitis in Japan in 1946. *Am. J. Hyg.*, **51**: 1–12.
3. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., and McClure, H. E., 1959a. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses of birds. *J. Immunol.*, **83**: 605–613.
4. Buescher, E. L., Scherer, W. F., Grossberg, S. E., Chanock, R. M., and Philpot, V. B., Jr., 1959b. Immunologic studies of Japanese encephalitis virus in Japan. I. Antibody responses following overt infection of man. *J. Immunol.*, **83**: 582–593.
5. Casals, J. and Brown, L. V., 1954. Hemagglutination with arthropod-borne viruses. *J. Exp. Med.*, **99**: 429–449.
6. Scherer, W. F., Buescher, E. L., Flemings, M. B., Noguchi, A., and Scanlon, J., 1959a. Ecologic studies of Japanese encephalitis virus in Japan. III. Mosquito factors. Zootropism and vertical flight of *Culex tritaeniorhynchus* with observations on variations in collections from animal baited traps in different habitats. *Am. J. Trop. Med. & Hyg.*, **8**: 665–677.
7. Scherer, W. F., Kitaoka, M., Grossberg, S. E., Okuno, T., Ogata, T., and Chanock, R. M., 1959b. Immunologic studies of Japanese encephalitis virus in Japan. II. Antibody responses following inapparent human infection. *J. Immunol.*, **83**: 594–604.
8. Scherer, W. F., Moyer, J. T., and Izumi, T., 1959c. Immunologic studies of Japanese encephalitis virus in Japan. V. Maternal antibodies, antibody responses and viremia following infection of swine. *J. Immunol.*, **83**: 620–626.
9. Southam, C. M., 1956. Serological studies of encephalitis in Japan. II. Inapparent infections by Japanese B encephalitis virus. *J. Infect. Dis.*, **99**: 163–173.

ECOLOGIC STUDIES OF JAPANESE ENCEPHALITIS VIRUS IN JAPAN.

IX. Epidemiologic Correlations and Conclusions*

E. L. BUESCHER and W. F. SCHERER

Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, U. S. Army, Japan

Study of the ecology of Japanese encephalitis (JE) virus in several habitats near Tokyo between 1952 and 1955 established that infections of the *vector* mosquito, *C. tritaeniorhynchus*, the wild avian and porcine *amplifying* hosts, and the *diseased* host, man, were annual late summer phenomena. As the study progressed, it was postulated that infections of these hosts were ecologically related, for they occurred almost simultaneously (mosquitoes, birds and pigs) or after a 2-week interval (man) (Articles II, IV, VI and VII). Further, the titers and duration of viremia in laboratory infected birds[1] were adequate to infect 47 to 93% of *colonized C. tritaeniorhynchus* and, after extrinsic incubation of 15 or more days, 43 to 92% of infected mosquitoes would transmit to birds and pigs.[2] The quantitative dynamics of mosquito-bird-swine infections, and their epidemologic significance to human infections, however, required further study. During the summer of 1956, infections of these hosts were investigated concurrently in Shinhama and Sagiyama, two separate sites in the Kanto plain (Article I). By correlating the times and extents of mosquito and vertebrate infections, a sequence for natural summer dissemination of JE virus was developed; through study of their population dynamics, natural behavior, and knowledge of their relative ability to infect, and be infected by *C. tritaeniorhynchus*,[2] the relative importance of each host to virus dissemination could be evaluated. For convenience, this correlation of results divides the natural infection cycle of JE virus into the three periods of *pre-emergence*, *virus dissemination*, and *declination to eclipse*.

Between April and late June, the *pre-emergence* period, JE virus is undetectable by the methods used in selected habitats of the Kanto plain. However, the vector, *C. tritaeniorhynchus*, terminates hibernation and begins to breed during this interval and over-wintering and migratory birds nest and begin to produce young. On the other hand, populations of swine are relatively constant during this period since, by deliberate design, farrowing of domestic sows is completed by April, and pigs are slaughtered in large numbers only in June and September. By late June total populations of *C. tritaeniorhynchus* have attained maximal levels. The *pre-emergence* period ends when JE virus is detected initially in the vector as early as 2 to 3 weeks before the first avian and porcine infections.

During the next interval, *virus dissemination* (July to late September), total vector populations decline as mosquito breeding decreases. Yet this apparently does not prevent further virus dissemination among the large numbers of susceptible birds and pigs, since their infections increase in frequency. Once virus appears in mosquitoes, it transfers rapidly and efficiently between vector and amplifier hosts until the density of infected mosquitoes becomes great enough in August to effect transmission of virus to man. To document these points, the period of virus dissemination in 1956 is illustrated in Figure 1. Virus first became evident in *C. tritaeniorhynchus* during late June. Even though the total vector population was maximal in late June and early July, the *infected* vector population did not reach peak densities until 1 month later. Infection of the amplifying hosts, birds, and pigs, increased in frequency during late July and early August, lagging several weeks behind vector infection. Equine disease, though relatively infrequent and ecologically unimportant (Article VI), was concurrent with infection of swine. Proved human infection in the same habitat was the last event in this sequence, probably because man is less frequently attacked by the vector than pigs or birds (Article III) and therefore is not infected until the density of infected vectors is maximal. Peak occurrence of human encephalitis and related deaths in Tokyo also followed infection of amplifying hosts. The few reported cases and deaths in May, June and

* Presented in part before the Sixth International Congresses on Tropical Medicine and Malaria, Lisbon, September 1958.

THE EPIDEMIOLOGIC SEQUENCE OF INFECTIONS BY JAPANESE ENCEPHALITIS VIRUS IN 1956

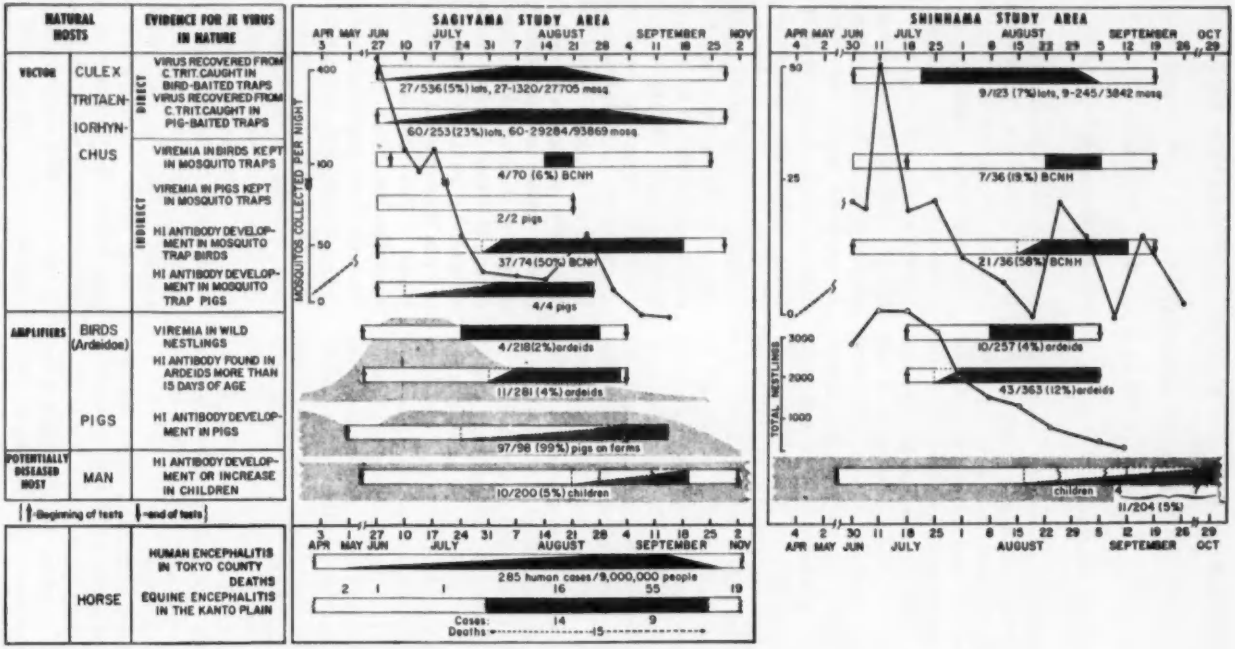


FIG. 1

July were unconfirmed by laboratory test as JE and probably were of other etiology (Article VII).

Of equal ecologic importance to the local spread of JE virus in the Shinhama and Sagiyama study sites are the mechanisms for the dispersion of virus from rural foci to areas of high population density such as Tokyo. Such transfer of virus over distances of 15 to 20 miles is difficult to explain solely by flight range of large numbers of infected *Culex* mosquitoes. Rather, it is more likely that JE virus is dispersed from infected habitats to areas devoid of virus through the daily movements of viremic birds, which could initiate secondary infection cycles in and near cities. In such secondary foci, relatively few new viremic birds and pigs could infect thousands of *C. tritaeniorhynchus* (Article III), and create a density of infected vectors sufficient to transmit virus to man.

In late September, new infections by JE virus *decline* in frequency, and virus is *eclipsed* until emergence the following summer. This eclipse is only partly explained by the behavior of amplifying hosts. By mid-September, swine are eliminated as effective virus amplifiers because they are either immune or slaughtered, and not replaced through breeding until mid-winter (Article VI). However, susceptible bird populations are never exhausted (Article IV). Therefore, it seems that the most probable explanation for cessation of virus dissemination each fall is the final disappearance of active vectors (Article II).

Obviously this description of the summer dissemination of JE virus is oversimplified since many aspects of virus, vector and vertebrate ecology can, and probably do, affect time and extent, though not necessarily sequence, of infection. For example, infected mosquitoes were first found in late June, 1956, at Sagiyama but not until late July at Shinhama, probably because of differences in vector and/or amplifying host populations. Yet the sequences of infection in each area were similar (Fig. 1).

The reasons for variation in size of epidemics and epizootics are unknown though there would appear to be at least two possible explanations. In certain years infected vector populations may be unusually large and transmit virus to extraordinarily large numbers of people and horses. In turn, the numbers of *diseased* hosts would increase in proportion to the numbers *infected*, assuming that a constant ratio exists each year between infected and diseased animals. The other explanation assumes that the ratio of infected to diseased hosts can change as virus strains of greater or lesser virulence appear in nature. Thus, epidemics might result from transmissions of highly virulent JE virus at a usual frequency rather than from increased transmission of ordinary virus.

Other large gaps also exist in understanding the ecology of JE virus. For example, it is still unknown how virus survives the winter in endemic areas of Japan. While the present studies were not designed to investigate this problem, it is logical to suggest that JE virus overwinters in hibernating, adult, female *Culex tritaeniorhynchus*, even though the numbers of overwintering mosquitoes are small. This thesis is supported by the high mosquito infection frequencies observed during these studies, and by the annual and consistently-timed recurrences of *C. tritaeniorhynchus* and JE virus at the study sites each year. It is possible, however, that alternate cycles as yet unidentified, involving ecto- or endo-parasites other than mosquitoes, and vertebrates other than birds or pigs, play a role in the interepidemic survival of JE virus. It is also possible that virus survives in latently infected warm- or cold-blooded animals, although such a mechanism would have to be consistent with regular annual reactivation of latent infection during early summer in areas where *C. tritaeniorhynchus* exists. Man and swine seem unlikely hosts for such latent infections although birds might be important in this respect since the phenomenon has been reported for birds and Western equine encephalitis virus.[3] No evidence has been obtained in the present studies to confirm or reject this hypothesis. Understanding of the overwintering mechanism(s) of JE virus will undoubtedly clarify the method by which virus emerges each summer. Although the most logical explanation for endemicity of JE resides in local overwintering of the virus, one cannot at this time completely eliminate the possibility of annual reintroduction of virus by migrating birds from southeast Asia (Article V).

The present study establishes that multiple hosts are involved in the natural dissemination of JE virus in Japan. This would suggest multiple points of attack in control of disease. Since all evidence points to a single mosquito species as the only important vector, it might seem logical to attempt control of the vector population. However, current methods for culecine mosquito

control could not be applied effectively in the Kanto plain without jeopardizing rice production and the local economy. Attempts to control dissemination of virus by reducing the effective population of susceptible amplifying hosts is not feasible, since pigs cannot be physically removed from the environment without seriously involving the basic economy, and significant reduction of the wild bird populations would currently be a monumental, expensive task. While it is possible that pigs could be artificially immunized to reduce the susceptible population, removal of but one major amplifying host probably would not affect the overall dissemination of virus, since the supply of the other amplifying host (birds) is never exhausted. By elimination therefore, it would seem that the only effective method of control of overt human JE resides in removing susceptible humans from the effective environment, either mechanically by screening from the vector, or biologically by immunization. While screening is a currently available method, it is expensive for the Japanese, is not consistent with the traditional design of their homes, and would require a continuous public educational program for effectiveness. Immunization by vaccination potentially offers the most effective means for long-term, mass protection of humans, though at this time a satisfactory vaccine has yet to be developed.

REFERENCES

1. Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., and McClure, H. E., 1959. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses of birds. *J. Immunol.*, **83**: 605–613.
2. Gresser, I., Hardy, J. L., Hu, S. M. K., and Scherer, W. F., 1958. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles from infected pigs and chicks to susceptible pigs and birds. *Am. J. Trop. Med. and Hyg.*, **7**: 365–373.
3. Reeves, W. C., Hutson, G. A., Bellamy, R. E., and Scrivani, R. P., 1958. Chronic latent infections of birds with Western equine encephalomyelitis virus. *Proc. Soc. Exp. Biol. and Med.*, **97**: 733–736.
4. Scherer, W. F., Moyer, J. T., and Izumi, T., 1959. Immunologic studies of Japanese encephalitis virus in Japan. V. Maternal antibodies, antibody responses, and anemia following infection of swine. *J. Immunol.*, **83**: 620–626.

RECOVERY OF HERPES SIMPLEX, SPONTANEOUS MURINE AND CHICK VIRUSES IN TESTS ON SPECIMENS FROM INFECTIOUS HEPATITIS PATIENTS

J. A. MORRIS* AND K. NAKAMURA

406th Medical General Laboratory, APO 343, San Francisco, California

This communication describes results of work performed during a 28-month period to determine, first, the incidence of viruses recoverable in a variety of experimental hosts in clinical materials obtained from young adults hospitalized with infectious hepatitis and, second, to assess the etiologic significance of the recovered agents in the patients' illness.

MATERIALS AND METHODS

Patients and collection of specimens. Patients used in this study were United Nations soldiers between the ages of 18 and 42 (average age 24) hospitalized with infectious hepatitis in the Far East during the period September 1956 to January 1959. Blood specimens for virus isolation and serologic study were collected in venules from patients usually on the 1st or 2nd day of hospitalization and additional specimens were obtained periodically thereafter for 1 to 6 months. Throat washings were obtained in veal infusion broth (containing 1000 units of penicillin and 0.1 mg of streptomycin/ml) at the same time as the initial blood specimens were obtained and stools were obtained as soon thereafter as possible. Liver tissues were obtained from fatal cases of infectious hepatitis within 24 hours after death. Throat washings, stools, and autopsy materials immediately after collection and serum after separation from the clot, destined to be employed in isolation studies, were stored in a CO_2-ice box at −70°C in rubber- or plastic-capped glass tubes or specimen bottles for periods of 7 to 60 days before being thawed for use. Sera for use in serologic work were stored at −20°C in plastic-capped glass tubes for 1 to 20 months. In addition, sera were collected at Willowbrook State School, Staten Island, New York, in 1956–57 from patients (3 to 10 years of age) immediately before and periodically after feeding of the Willowbrook strain of hepatitis virus[6] and at American military hospitals in Germany in 1957 from cases of naturally acquired hepatitis in American soldiers. These sera were also used in the present study in serologic tests and were kindly furnished to us by Drs. R. Ward, S. Krugman, and J. Paul.

Stools and autopsy specimens were triturated in a TenBroeck grinder with sufficient Hanks's balanced salt solution containing 1000 units of penicillin and 1 mg of streptomycin/ml to give 10 per cent suspensions. These suspensions, and thawed throat washings, were clarified by spinning at 2000 rpm for 30 minutes in a horizontal centrifuge. The supernatant fluids of this spinning constituted the inocula and immediately after preparation were injected into experimental hosts.

Experimental hosts. Test materials were examined for presence of virus which might be pathogenic for one or another of the 7 different hosts listed in Table 1. Cultures of human cells, derived from conjunctiva (strain Chang), liver (Chang), intestine (Henle), and bone marrow (obtained from Walter Reed Army Institute of Research), were grown in accordance with standard procedures[1] in stationary tubes and bottles in Hanks's balanced salt solution containing 20 per cent inactivated (56°C for 30 minutes) normal horse serum. At the time of use (4 to 6 days after seeding) in virus isolation procedures and in preparation of complement fixing antigens and seed viruses, the growth medium was replaced in bottles with 20 ml and in tubes with 1 ml of maintenance solution consisting of a mixture of 199 medium and 2 per cent inactivated horse serum. Swiss mice were obtained from a commercial dealer and were from noninbred stock originating from animals brought to Japan from Germany about 30 years ago. It was known that in this stock ectromelia and hepatoencephalitis were enzootic. Chicks were Leghorns purchased from a dealer who reported that except for pullorum disease no enzootic illnesses had been

* Present address: Division of Biologics Standards, National Institutes of Health, Bethesda 14, Maryland.

TABLE 1

Number of hepatitis specimens examined in experimental hosts for agents of transmissible illness

Specimen	Total number examined	Experimental hosts						
		Cell cultures				Suckling Mice	Chick embryos	Baby chicks
		Bone marrow	Liver	Intestine	Conjunctiva			
Stools	150	137	147	145	142	137	22	0
Throat washings	100	77	46	43	88	30	25	0
Sera	150	37	47	45	42	137	7	93
Autopsy materials	5	5	3	5	4	2	2	1
Total isolation attempts	405	256	243	238	276	306	56	94

noted in the flock in the last 5 years. At the time of use mice and chicks were 1 to 5 days old. Embryonated eggs from Leghorn chickens were purchased commercially and were injected on the 10th to 13th day of incubation.

Virus isolation. Each of 2 bone marrow, liver, intestine and conjunctiva culture tubes received 0.2 ml of test inoculum. In many instances, aliquots of the inocula were injected by multiple routes into suckling mice (intracerebrally and intraperitoneally), embryonated eggs (yolk sac and chorioallantoic membrane), and chicks (intracerebrally and intravenously). Inoculated cell cultures were incubated at 33 to 35°C in a stationary position or in a roller drum. Nutrient fluids were changed every 3 or 4 days. Daily examinations were made of inoculated cell cultures for 21 to 28 days, mice and chicks for 30 to 60 days and embryonated eggs for 6 to 9 days. In instances where cultures showed questionable or definite cytopathologic changes or animals developed evidence of illness, passages were undertaken to determine whether or not the observed changes were serially transmissible.

Serology. All transmissible agents encountered in inoculated cell cultures and in test animals were first examined for etiologic importance in human hepatitis by neutralization and complement fixation tests using paired serums from hepatitis patients and culture fluids or animal tissues infected with 2nd to 4th passage of the agent in question. The H16 strain of herpes simplex virus, 307 strain of ectromelia, and 4 strains of an unidentified agent associated with a transmissible hepatitis of baby chicks employed in these tests, were recovered during the current studies in this laboratory. The H747 virus employed as prototype strain of murine hepatoencephalitis virus was isolated in 1953 in mice in Tokyo during studies concerned with Far East hemorrhagic fever.[5] Neutralization tests were performed in mice inoculated with H16, 307 and H747 viruses in the usual manner employed in U. S. Army Laboratories[3] using one or both of the virus and serum dilution techniques. No satisfactory neutralization test was developed for the chick hepatitis agent. Complement fixation tests were performed in the standard way[3] using antigens prepared from human cell cultures infected with H16 strain of herpes simplex or from brains of suckling mice infected with H747 strain of hepatoencephalitis virus or with the 307 strain of ectromelia virus and from livers of chicks infected with one or the other of two strains of the chick hepatitis agent. Transmissible agents which were neutralized or which fixed complement with convalescent hepatitis serums were further tested to determine their identification. Agents not neutralized by hepatitis serums were, where possible, also identified.

RESULTS

Twenty-eight agents of transmissible disease were recovered in experimental hosts inoculated with one or the other of 405 clinical specimens obtained in the acute phase of illness from 200 hepatitis patients. Types of specimens and hosts into which they were inoculated are given in Table 1.

Agents recovered in mice. Seventeen of the 28 transmissible agents were recovered in inoculated mice: 12 of these were identified as murine hepatoencephalitis virus and 5 were shown to be mouse pox virus. In neutralization tests with prototype strains of murine hepatoencephalitis (strain H747) and mouse pox virus (strain 307) none of the paired sera from 6 hepatitis patients contained in a 1:1 dilution demonstrable antibody against the hepatoencephalitis virus; however, all sera, both acute and convalescent specimens, from all 6 patients contained substances which neutralized 2 and 3 logs of mouse pox virus. The latter finding is in agreement with the observa-

tion that neutralizing antibody in human sera resulting from Jennerian vaccination is capable of neutralizing other members of the mammalian pox group of viruses, including ectromelia.[4] From these observations it was assumed that all 17 viruses recovered in mice were contaminants derived from this experimental host. Results of complement fixation tests supported this assumption. In examinations of paired acute and convalescent hepatitis serum from 6 patients and antigens prepared from normal mouse liver and from livers of mice infected with hepatoencephalitis or ectromelia viruses, no specific fixation was obtained. At serum dilutions 1:8 and 1:16 complement was fixed in presence of both test antigens but these sera also fixed complement in the presence of control antigen.

Agents recovered in 1 to 5 day old chicks. Four agents of transmissible disease were recovered in chicks inoculated with hepatitis materials. These agents were associated with nonfatal disease characterized primarily by macroscopic focal necrosis of the liver. In subsequent work, similar lesions were found in chicks injected with suspensions of "normal" chick tissues. None of these agents consistently induced macroscopic lesions in all inoculated chicks; only 3 or 4 of 6 birds injected with low dilutions of infectious material were found 1 to 3 weeks later to have macroscopic liver lesions, and because of this property of the infectious agent, no satisfactory neutralization test procedure was developed. Efforts were made, however, to relate the chick agents to human hepatitis by application of the complement fixation technique. Paired acute and convalescent phase sera from 6 hepatitis patients were examined in complement fixation tests for antibody against antigens prepared from liver tissue of chicks free of macroscopic liver lesions and from chicks with liver lesions resulting from injection of strains of the chick hepatitis agent. All tests with hepatitis sera gave positive results at dilutions of 1:32 and 1:64 with test antigens, but these sera also fixed complement at the same dilutions with the control preparation. Thus, occurrence of an agent of transmissible hepatitis in control chicks, which by available techniques could not be distinguished from agents recovered in chicks inoculated with human hepatitis materials, together with failure to establish a positive serologic relation between the avian and human diseases, provided the evidence suggesting that the disease encountered in chicks in this work was an enzootic one unrelated to human hepatitis.

Virus isolation results in chick embryos. Results of isolation attempts in embryonated hen eggs were entirely negative. No transmissible agent was recovered in chick embryos inoculated by yolk sac and chorioallantoic membrane routes with any of the 56 hepatitis materials listed in Table 1. Furthermore, none of the extraembryonic fluids harvested 6 to 9 days after inoculation of the test materials contained agglutinins reactive with either chick or human "O" erythrocytes.

Agents isolated in human cell culture. Seven strains of herpes simplex virus were isolated in human conjunctiva cell cultures inoculated with throat washings from as many hepatitis patients. The 7 specimens which yielded herpes simplex virus in cell cultures were also examined in newborn mice and in chick embryos with negative results. None of 93 other hepatitis throat washings and none of 305 other hepatitis specimens yielded virus in any of the 4 human cell lines listed in Table 1. Patients from whom herpes simplex virus was recovered did not exhibit at any time during their hospitilization cutaneous lesions which suggested herpes simplex infection. Moreover, the clinical course in these individuals did not differ significantly from that observed in hepatitis patients from whom virus was not recovered.

In Tables 2 and 3 are shown results of serologic

TABLE 2

Occurrence of H16 CF antibody in paired sera from patients hospitalized with clinically diagnosed infectious hepatitis

Area	Total number cases	H16 antibody		Number showing H16 CF antibody with	
		Present	Absent*	No rise	4-fold or greater rise
Far East..	238	194 (82%)	44 (18%)	189	5
Willowbrook...	6	4 (67%)	2 (33%)	2	2
Germany..	22	15 (68%)	7 (32%)	14	1
Totals..	266	213 (80%)	53 (20%)	205	8

* In 1:8 dilution of serum the lowest dilution tested.

TABLE 3

Findings in 15 cases of infectious hepatitis in whom positive evidence of concurrent infection with herpes simplex was obtained

Geographic area	Patient	Date	Day after onset of jaundice	Serologic findings*		Virus isolation (throat washing)
				CF titer	Neutral titer	
Far East	1	1/25/57	11	0	2	Neg
		4/30/57	106	16	12	
	2	8/20/57	17	0	ND	Neg
		9/18/57	46	8	ND	
	3	5/22/57	19	0	2	Neg
		6/19/57	47	64	33	
	4	7/23/57	13	0	2	Neg
		9/18/57	70	32	16	
	5	7/11/57	?	0	2	Neg
		7/23/57	?	32	20	
	6	1/11/58	7	16	10	Pos
		2/3/58	30	32	16	
	7	7/5/57	7	32	40	Pos
		7/18/57	20	64	80	
	8	1/9/58	10	64	20	Pos
		2/25/58	57	32	10	
	9	7/17/57	11	64	24	Pos
		8/27/57	52	32	6	
	10	1/10/58	9	64	40	Pos
		2/14/58	44	64	40	
	11	1/10/58	7	32	100	Pos
		2/4/58	32	32	100	
	12	1/9/58	7	128	16	Pos
		2/11/58	40	128	16	
Willowbrook	13	11/27/56	−66	0	ND	ND
		3/22/57	50	32	ND	ND
	14	2/25/57	−64	8	ND	ND
		6/6/57	37	128	ND	ND
Germany	15	8/5/57	A	0	ND	ND
		9/2/57	C	8	ND	ND

* ND = not done
A = acute specimen
C = convalescent specimen

tests with paired sera from hepatitis cases occurring in United Nations soldiers in the Far East and a prototype strain (H16) of the recovered herpes simplex viruses. Use of serologic tests resulted in detecting 4-fold or greater increases in H16 antibody in 5 of 238 Far East cases examined, but, as is seen in Table 3 (first 5 patients listed), from none of this group of 5 patients was virus recovered. Nevertheless, the serologic findings on these individuals would seem to indicate that all 5 patients, while hospitalized with clinically diagnosed infectious hepatitis, experienced primary infection with herpes simplex virus.[3] Serologic examination of the 7 patients from whom virus was recovered resulted in detection of significant herpes simplex antibody rises in none (patients 6 to 12 in Table 3). These 7 patients, at the time virus was recovered from their throats, possessed in their serum high levels of complement fixing and neutralizing antibodies directed against herpes simplex virus. This finding reflects the probable existence in these patients of latent herpetic infection.[3] Altogether, virologic and serologic evidence indicating that infection with herpes simplex virus was concurrent with clinical manifestations of infectious hepatitis was obtained in 12 of 238 Far East hepatitis patients.

Tests were next performed to determine the incidence of active infection with herpes simplex virus in patients hospitalized with infectious hepatitis in other areas of the world. Examination in complement fixation tests of paired sera obtained from children with experimentally induced hepatitis in the United States[4] and from soldiers with naturally acquired hepatitis in Germany showed that 2 of 6 United States patients and 1 of 22 German cases developed in the course of their clinical hepatitis 4-fold or greater rises in serum antibody titer to herpes simplex virus (See Table 2 and 3). Table 2 also shows the breakdown for patients for whom no herpes simplex complement fixing antibody was demonstrated. It is seen that approximately 20 per cent (53) of 266 hepatitis patients in convalescence did not possess detectable serum levels of this antibody.

DISCUSSION

Our purpose was to examine systematically clinical materials obtained from infectious hepatitis patients for a transmissible agent which might be pathogenic for one or the other of several experimental hosts and, further, to assess the etiologic significance of the recovered agent in the patient's illness. We examined in virus isolation or in serologic tests 260 cases of naturally acquired infectious hepatitis in the Far East and in Germany and 6 cases of experimentally induced hepatitis in the United States. In this work 21 strains of viruses were recovered in animal hosts; these viruses require no comment since each was clearly a contaminant unrelated in any demon-

strable way to the human disease, infectious hepatitis.

The exhaustive isolation studies made in the current work have established that the preponderance of our hepatitis patients was strikingly free of viruses recoverable by application of the isolation techniques currently in use. Thus, from not a single patient in a series of 200 was a virus, other than herpes simplex, recovered in any of 7 experimental hosts including cultures of 4 different human cell lines. Failure to isolate enteric viruses from throat washings and fecal specimens or circulating viruses from the sera obtained from such a large sampling of acutely ill hepatitis patients was not referable to isolation techniques employed since these viruses were concurrently recovered in the same laboratory in routine isolation procedures. Instead, the negative results suggest absence in throats, feces, and sera of the patients under study of viruses recoverable by the techniques employed.

The virologic and serologic evidences of infection with herpes simplex virus among hepatitis patients are not wholly compatible with expected findings based on previous experience with normal individuals. Older observations on incidence of detectable complement-fixing antibody against herpes simplex virus in United States military personnel and their dependents in the age group 20 to 40 years show that about 50 per cent possess this antibody in demonstrable amounts.[2] The significance, if any, of the greater incidence of herpes simplex complement-fixing antibody (80 per cent) in our series of hepatitis patients is not known, but might be due to activation in positive patients of latent infection under such conditions that intensity of infection is sufficient to result in production of complement fixing antibody. Such an explanation, however, hardly fits the serologic findings in patients who experienced a 4-fold or greater increase in herpes simplex complement-fixing antibody in the course of their clinical hepatitis. In these patients the increase in complement-fixing antibody between the acute and convalescent specimens is regarded as compatible with a *primary* infection with the virus of herpes simplex.

All in all, we are inclined to believe that a reasonable explanation for the observations of active herpes simplex infection in hospitalized infectious hepatitis patients in our series is that infections with herpes simplex virus and the agent of infectious hepatitis occurred simultaneously and possibly independently of each other in the same patient. The observations on 5 patients considered to have had primary herpetic disease are not as easily explained, but here again, we are of the opinion that these cases represent dual infections.

SUMMARY

Inoculation into 1 or more of 7 experimental hosts of 405 clinical specimens obtained from 200 infectious hepatitis cases resulted in recovery of a number of enzootic viruses unrelated in any demonstrable way to the human disease, and in isolation from 7 of 200 patients of herpes simplex virus. Serologic study of 259 other hepatitis patients revealed concurrent hepatitis and herpes simplex infections in an additional 8 patients. The evidence that these 15 patients were suffering dual infections is presented and discussed.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to Col. D. Schamber and L. Saylor, Deputy Surgeons, 8th U. S. Army; to Col. A. L. Tynes, Commanding Officer, Lt. Col. R. L. Gale, Assistant Chief, Medical Service, Capt. R. E. Dunlap and 1st Lt. D. J. Beach, Clinical Laboratory Officers, U. S. Army Hospital, Zama, Japan; to Col. P. J. Smith, Chief, Department of Medicine and Lt. Col. S. M. Dozier, Chief, Laboratory Service, Tokyo Army Hospital, Tokyo; to Majors T. G. Nelson and J. Keeton, Commanding Officer and Chief of Medicine, respectively, Capt. L. A. Rosenblum and 2nd Lt. H. C. Phelps, Laboratory Officers, 121st Evacuation Hospital, Seoul, Korea; Dr. R. J. Truex, Marine Aircraft Group 11, Pingtung, Formosa; Capt. R. H. Philips and Dr. J. T. Grayston, NAMRU 2, Taipei, Taiwan; Col. H. Marshall, Director, Public Health and Welfare Department, Ryukyu Islands; Col. E. A. Hefferman, Commanding Officer, and Major J. Metzger, Chief, Laboratory Officer, Ryukyus Army Hospital; Lt. Col. A. C. Buchanan, Commanding Officer, 43rd Surgical Hospital, Korea; and to members of their staffs for cooperation in obtaining and in preparing specimens used in this study. To Brig. Gen. J. H. McNinch, Surgeon, U. S. Army, Japan, Col. C. H. Moseley, Commanding Officer, Army Medical Center, Japan, and Col. M. W. Bayliss, Commanding Officer, 406th Medical General Laboratory, we wish to express our thanks for making it possible to carry out this study.

In this work, at one time or another, valuable technical assistance which is gratefully acknowledged was kindly given by R. P. J. Smith, M. Tanabe, J. M. McCown, L. Allen, R. Walters, T. Misiewicz and R. Blount. Mrs. Pari Morse kindly prepared for use in this study cultures of human cells and assisted in the performance of neutralization tests.

REFERENCES

1. Chang, R. S., 1954. Continuous subcultivation of epithelial-like cells from normal human tissues. *Proc. Soc. Exper. Biol. & Med.* **87:** 440–443.
2. Dascomb, H. E., Adair, C. V., and Rogers, N., 1955. Serologic investigations of herpes simplex virus infections. *J. Lab. & Clin. Med.* **46:** 1–11.
3. Department of the Army and Airforce, 1951. Methods for medical laboratory technicians (TM 8-227. AFM 160-14) Chapter 11, Rickettsiae and Viruses, Washington, D. C.; U. S. Government Printing Office, 523–558.
4. Fenner, F., 1949. Mouse-pox (infectious ectromelia of mice): A review. *J. Immunol.* **63:** 341–373.
5. Morris, J. A. and Aulisio, C. G., 1954. A new member of hepatoencephalitis group of murine viruses. *Fed. Proc.* **13:** 506.
6. Ward, R., Krugman, S., Giles, J. P., Jacobs, A. M., and Bodansky, O., 1958. Infectious hepatitis: Studies of its natural history and prevention, *N. England J. Med.* **258:** 407–416.

BOOK REVIEWS

Perspectives in Virology, by MORRIS POLLARD, University of Texas, Medical Branch. 312 pages, illustrated. New York, John Wiley & Sons, Inc., 1959. $7.00.

A symposium entitled "Perspectives in Virology" was held in 1958, in which an attempt was made to assess recent progress in various virologic fields and to project future pathways of research in this area. The monograph edited by Dr. Morris Pollard presents the formal papers and a transcription of the extemporaneous discussions of these papers by a group of leading virus workers. The subjects considered covered a wide range of topics at the forefront of current virus research. Events at the virus-cell level concerned with the chemical nature of virus infectivity, genetic interaction between bacteriophage and host bacteria, genetic recombination among pox viruses, the mechanism of myxovirus replication, the nutritional requirements for virus propagation, and cell plating techniques as a tool for the quantitative measurement of virus action, are discussed by workers who are leaders in their fields. Topics of importance to infectious disease epidemiology and preventive medicine are given excellent coverage in the presentations dealing with newer knowledge of measles virus, implications of the newly recognized viruses of man, the natural history of hog cholera, specific humoral immunity as a factor in virus disease ecology, and problems in the epidemiology of Asian influenza. Finally, current status and prospects of virus neoplasia in animals are ably discussed by workers who have made leading contributions in this field, which presently appears to be in an exponential growth phase.

No attempt is made to provide a comprehensive coverage of all current virologic problems. The areas selected, however, are those in which the most rapid current advances have occurred and in which future advances of a broader nature appear equally probable.

ROBERT M. CHANOCK

The Mammals of North America, by E. RAYMOND HALL, Ph.D., Chairman of the Department of Zoology, University of Kansas, and KEITH R. KELSON, Ph.D., Division of Scientific Personnel and Education, National Science Foundation. 2 vols., 1,373 pp., 1,216 illustrations. New York, the Ronald Press Co., 1959. $35.00.

This monumental work provides a synopsis of the taxonomy and distribution of mammals of North America, including Greenland, south to Panama and to Grenada in the Lesser Antilles. Orders, families, genera and species are characterized compactly, and selected facts on their biology are summarized at the appropriate rank. Keys to families, genera, and species are provided, and taxa of other ranks from sub-orders down (but not subspecies) are included in the keys if they figure in the taxonomic scheme adopted for a given group. Critical citations supporting the adopted nomenclature, binomial or trinomial, as the case may be, are given in conventional manner. Exclusive of marine mammals, each species, with its collective subspecies if any are recognized, is provided with a distribution map; marginal records are shown there by dots and then listed and documented in text. Otherwise, subspecies are merely listed alphabetically. In addition to 500 maps, there are 538 figures of skulls exemplifying most genera and species, and also many unnumbered ink drawings of mammals including at least one representative of most genera. Complete indices to vernacular and technical names used in both volumes appear at the ends of both volumes. Other than preface describing the organization of the work, the only sections of straight text are an 8-page discussion of zoogeography and a 12-page section of suggestions for collecting and preparing specimens.

By comparison with Miller and Kellogg's "List of North American Recent Mammals" (U. S. Nat. Mus. Bull. 206), which appeared in 1955, this work provides more information, although the statements of ranges in the former are replaced by maps with lists of marginal locality records in the latter. Hall and Kelson's handling of taxonomic categories is not so conservative as that of Miller and Kellogg. Thus, the former recognize 1003 species, the latter, 1065. The difference at the generic level is in the same direction. Other kinds of differences occur (including the inevitable residue having to do merely with nomenclatorial technicalities); but altogether, the two classifications are very similar.

Of routine points raised by reviewers of regional taxonomic works, such as regrettable or debatable changes in names, or errors in details of distribution maps, examples detected by me or called to my attention in this large work are comparatively few. Editorial slips, likewise, are few. More serious, since these books will be used by many who are not primarily specialists in mammalogy, are certain debatable features of mapping procedure. Thus, for poorly known Mexican and Central American species, the distribution shown often sweeps across large gaps without records and without regard for regional topographic and ecologic zones surely indicating absence. Also, for closely related allopatric species which may or may not be conspecific, the maps show their distributions abutting when one wants to know of any record of marginal overlap which would indicate

existence of genetic as well as lesser barriers to interbreeding. Moreover, this abutting of neighboring distributions carries implications about their dynamics which are not only unsupported, but biologically unlikely and hence objectionable. The underlying drive for this work would seem to be an interest in the multiplicity of "kinds" of mammals (species plus subspecies) to be found in a given area, with the result that much if not most of the effort was devoted to delineation of subspecies, when actually the lasting and more widely appreciated values will derive from the information on species *per se*. Hence, a greater emphasis on species limits, in mapping and otherwise, was in order. In any event, in this decade of enlightenment about speciation processes, something more positive could have been done by the authors in treating the ludicrous six dozen species of brown bears named by C. Hart Merriam. But, accepting their cautions in use of the species concept, then in this as in other works of Dr. Hall and his colleagues, one is impressed by the painstaking care given to compilative and mechanical detail. No similar set of maps has been available previously for North American mammals, and to me they represent the most significant distillate of the enormous effort behind this work. For any biologist interested in taxonomy, distribution, ecology and evolution of American mammals, in whatever way, these maps and the volumes housing them are a rich and indispensable source of critical information.

FRANK A. PITELKA

Annals of the Institute of Tropical Medicine. Special Number Dedicated to the VI International Congresses of Tropical Medicine and Malariology (in Portuguese and English), by various authors. Volume 15, Supplement 1, 510 pages, illustrated. Lisbon, Institute of Tropical Medicine, 1959.

The scope of this special historical number of the Annals is well expressed in the foreword by the Director of the Institute; (page 9) ". . . as the Institute of Tropical Medicine is the Body entrusted with the honor of organizing the Congresses, it is understandable that it should communicate to this important meeting, not only its work and activities during its 56 years of existence, but also all the disinterested efforts which the Country has made in the Overseas Provinces in the way of medical assistance to their inhabitants . . . this number of the Annals serves to tell what Portugal has achieved in altruistic medical services in the tropical regions; it begins with a short history of the life and work of the Institute of Tropical Medicine . . . special reference is made to the Research Institutes and Permanent Missions which under its orientation, operate overseas, and which, together with the Institute, constitute a working unit in the Country, whose homogeneity is the basis of its strength and action.

"A consideration of the medical bodies follows, beginning with the Commission of Hygiene and Health, Organization of the Overseas Health Services, followed by a description of the Overseas Hospital and work carried on by the Health Services of each of the Overseas Provinces.

"Finally a description is given of the contribution made by the Portuguese to Tropical Medicine Research. . . ."

The authors are to be congratulated on the results of the effort to bring together at the time of the Congresses, which coincided with the inauguration of the Institute's beautiful new building, all of the information regarding the development and activities of the Institute. Especially pleasing is the presentation in English, as well as Portuguese, although there are some typographical errors in English, so unavoidable where proof must be read in a language other than that of the country.

The information presented is most detailed as related to the staff and the graduates of the Institute from the beginning over half a century ago. A complete bibliography is given of all articles published on scientific work undertaken by staff members or under the auspices of the Institute, together with a list of over 1,100 scientific periodicals to be found in the Library.

Many references are found to specific diseases, covering points of historical and geographical interest. Unfortunately, these are not freely accessible, since this number of the Annals has not been indexed. (The rearrangements of the Table of Contents, and their publication at the end of the text as Indexes by Subjects and by Authors, does not meet the need for an index.) It is to be hoped that a future number of the Annals will bring a detailed index, which will make this valuable compilation more readily accessible to workers everywhere.

FRED L. SOPER

Antibiotics Annual 1958–1959, by HENRY WELCH, Ph.D. and FELIX MARTI-IBANEZ, M.D., editors. 1107 pages, illustrated. New York, Medical Encyclopedia, Inc., 1959. $12.00.

This volume contains the Proceedings of the Sixth Annual Symposium on Antibiotics held in the Fall of the year in Washington, D. C. It is, in 1,100 pages, a repository of much information about antibiotics, some new, some old, some of high quality and much of it pedestrian. There are papers on sulfonamides, nitrofurans, quaternary compounds, salicylic derivatives, oleandomycin,

triacetyloleandomycin, penicillin G, penicillin V, spiramycin, tetracycline, chlortetracycline, oxytetracycline, leucomycin, erythromycin, propionyl erythromycin, novobiocin, ristocetin, kanamycin, vancomycin, amphotericin B, and others. There is a series of papers on antitumor antibiotics.

Because of the size and scope of the Symposium itself, it has been difficult over the years to separate wheat from chaff. We find such hard-to-excuse papers as one which deals with sensitivity studies of antibiotics but does not define sensitivity and resistance as conceived by the author, a provocative study of the treatment of the common cold by "two tablets" without a statement as to the size of the tablets, and similar careless presentation of material. The clinical evaluations of drugs are particularly difficult to appraise. We live in a time when not only are new antibiotics being developed, but in forms which have apparent pharmacological advantages over older forms, or greater freedom from toxicity. It has become more and more difficult for the clinical investigator to compare these antibiotics with the older ones. As a result, a good deal of uncritical work is presented, often out of a feeling of obligation for a grant or for the donation of quantities of drug. For example, in one study of a new antistaphylococcal drug in outpatients the authors report almost 100% excellent results, but either did not think it necessary or did not think it proper to alternate patients treated with drug and wet dressings, etc., with those treated with wet dressings alone.

One excellent group of papers had to do with the standardization of discs used for antibiotic sensitivity tests. Standardization has been accomplished in Canada, where discs must be certified by a government agency prior to sale, but in this country we still permit the marketing of discs which often do not contain the amount of antibiotic stated on the label. Attention to such matters as the paper from which the disc is made, and the method by which the antibiotic is added, seems to be important. However, we shall continue to have to contend with nonuniform products in this highly competitive field until the government takes a hand in the matter.

One of the best features of the annual symposia have been the panel discussions, and 1958–59 is no exception. An excellent symposium on the current status of newer antibiotics, with particular reference to their use in staphylococcal disease, and one on the causation, prevention and control of staphylococcal disease in hospitals, were of high quality and are presented verbatim.

Another feature of this meeting was a series of addresses by Sir Howard Florey, Dr. Selman Waksman, Dr. Chester Keefer and Dr. Harry Dowling. All of these carry provocative messages, not the least of which was Sir Howard Florey's comment that he had only one serious regret about his experience with penicillin. The regret was that he did not, on behalf of his colleagues and the laboratory, patent the processes by which penicillin was extracted. He went on to say "This, looking back on it, was a cardinal error, but at that time the patenting of additional substances by medically qualified people was heavily frowned upon both in Great Britain and the United States. However, if the processes of extracting penicillin had been patented it would have saved me a great deal of worry in subsequent years for it seems to me that only by having available funds obtained by this means is it possible in Great Britain, at the present time, to be sure of keeping tried research workers and providing with certainty the income, ty, and fsecuriacilities which first-rate people in their thirties are surely entitled to. My department has, of course, been generously helped from many sources, for example by Lord Nuffield, the Albert and Mary Lasker Foundation, the Rockefeller Foundation, and drug firms of the United States and Great Britain. Nevertheless, I now find it almost impossible to find stable jobs for admirable young research workers in my own laboratory. They are the next generation, and I must confess that I feel rather frustrated in not being able to run a department so as to give positions and security to workers of promise." This situation is not confined to Great Britain.

MORTON HAMBURGER, M.D.

CORRECTION

In the article "Studies on Immunity to *Schistosoma Mansoni*—II. The Circumoval Precipitin Reaction to *S. Mansoni* in Mice Treated with Stibophen", on page 565 of the September, 1959 issue, volume 8, number 5, two illustrations were incorrectly placed.

The illustration identified as figure 1 on page 566 should be figure 2. The illustration identified as figure 2 on page 507 should be figure 1.

NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene. This is the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which will entail a certain delay in their appearance because of the contractual limitation on the number of journal pages available in any year. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

Publication will be facilitated if the following suggestions are followed:

1. *Manuscripts* should be typewritten in *English* using *double spacing* on *one side* only of 8½ x 11 inch paper and submitted *in duplicate*. Double-spacing should be used throughout the manuscript including the list of references, footnotes, and quotations.

2. The author should supply, on a separate sheet, a short title not over 50 letters for use as a running head. Also on separate sheets type (a) the authors' names and affiliations; (b) tabular matter; (c) case histories; (d) quotations; (e) formulas and other subsidiary matter in the text; (f) footnotes; (g) references; (h) legends for illustrations; (i) displayed formulas occupying more than 1 line of type, even if using the same size type as the main text.

3. Legends should be separate and not attached to, or written on, the illustration copy.

4. Positions for tables and figures in the text may be indicated on the margin of the text pages.

5. All papers should end with a brief summary.

6. *Tables* should be typed on separate sheets and be serially numbered in Arabic numerals.

7. *Figures* and *photographs* should be protected with cardboard to avoid creasing in the mails. Only glossy prints can be used. Line drawings and graphs should be made with India ink and submitted on white (not cross-section) paper. Colored illustrations can be reproduced only at the expense of the author.

8. References should be cited by consecutive numbers in the text, and so numbered and listed in the list of references at the end of the article, without regard to alphabetical arrangement. References should be written as follows:

> Reed, L. J., and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, 27 : 493–497.

9. *Proprietary names* of drugs or chemicals registered by commercial pharmaceutical firms may not be used in the text if there is a generic name available in the U. S. Pharmacopoeia, New and Nonofficial Remedies of the A. M. A., or the latest editions of standard medical dictionaries. In certain cases where the generic name is relatively unknown, the registered name may follow it in parentheses in the title, at the first citation in the text, and in the conclusions.

10. The address to which proofs are to be sent should be indicated at the top of the first page of the manuscript. Delay will result if proofs must be forwarded.

Estimating costs of reprints. Where the cost of reprints must be known in advance of publication in order to secure prior approval for their purchase, the Editor will be glad to furnish an estimate of the number of pages which the article will occupy in the Journal.

DIFCO LABORATORY PRODUCTS

BIOLOGICS CULTURE MEDIA REAGENTS

Media for Standard Methods

Culture Media *Dehydrated and Prepared*

Microbiological Assay Media

Tissue Culture and Virus Media

Bacterial Antisera and Antigens

Diagnostic and Serological Reagents

Sensitivity Disks Unidisks

Peptones Hydrolysates Amino Acids

Enzymes Enrichments Dyes Indicators

Carbohydrates Biochemicals

DIFCO

over 60 years' experience
in the preparation of Difco products assures
UNIFORMITY STABILITY ECONOMY

•

Complete Stocks *Fast Service*

•

Descriptive literature available on request

DIFCO LABORATORIES **DETROIT 1 MICHIGAN USA**